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UNIVERSITY OF ALBERTA

Processing of UV-Induced DNA Damage in Diverse Biological Systems

by

Anne Maxine Galloway ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Department of Genetics

Edmonton, Alberta
Fall, 1992

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FACULTY OF GRADUATE STUDIES AND RESEARCH

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Date 27th Aug. 1992

DEDICATION

This thesis is dedicated to Ken, who is undoubtedly the most precious gift God has given me, and to my parents, Lloyd and Violet, who didn't give up on me through my terrible twenties and continue to be my best friends.

ABSTRACT

A novel protocol has been developed which allows direct evaluation and accurate quantitation of UV lesions contained within both genomic DNA and the small oligonucleotides excised by a living cell during nucleotide excision repair. Using this methodology, the repair capacity of normal and UV-sensitive cells of human, Chinese hamster, and *Escherichia coli* origin, has been assessed. Several conclusions have been reached from these studies. Firstly, severage of the interpyrimidine phosphodiester linkage of cyclobutane dimers appears to be an evolutionarily conserved phenomenon. Secondly, the kinetics of cyclobutane dimer repair differ markedly from both (6-4) photoproduct and TA* lesion removal. The latter two classes of photolesions are removed rapidly from normal cells which, in mammalian cells, accounts for ~80% of unscheduled DNA synthesis at 2 hr post-UV. Thirdly, the ability to excise cyclobutane dimers is independent of (6-4) photoproduct repair capacity, suggesting that these lesions are not repaired/recognized by identical mechanisms. Fourthly, fibroblast strains representing the eight xeroderma pigmentosum complementation groups each show a unique proficiency/deficiency to repair the different photolesions under study, implicating that a defect in a different locus underlies each genetic form of this disease. Finally, the repair deficiency in UV-sensitive strains of trichothiodystrophy appears to be completely unrelated to that of non-complementing XP-D cells, challenging the relationship of these defects.

To allow direct assessment of an IDP-altered photoproduct, substrates have been constructed which contain, at a defined dithymidine site, no lesion, a conventional cyclobutane dimer, or a cyclobutane dimer modified by severage of the intradimer phosphodiester bond. These constructs have been utilized to assess the behavior of various enzymes upon encountering a cyclobutane dimer with a cleaved versus intact intradimer phosphodiester linkage. Bacteriophage T4 UV endonuclease has no activity towards a modified lesion, questioning the interpretation of experiments which utilize the strand-incising activity of this enzyme to monitor repair. Furthermore, although this altered lesion acts as a block to *E. coli* DNA polymerase I, it allows SP6 RNA polymerase to bypass the otherwise RNA polymerase-blocking lesion.

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TABLE OF CONTENTS

CHAPTER ONE - AN OVERVIEW 1

CHAPTER TWO - REPAIR OF CYCLOBUTANE DIMERS AND (6-4) PHOTOPRODUCTS IN NORMAL HUMAN FIBROBLASTS: USE OF A NEW METHODOLOGY TO MONITOR THE REPAIR OF UV-INDUCED DNA DAMAGE 10

- INTRODUCTION 10

- EXPERIMENTAL PROCEDURES 14
  - Materials. 14
  - Cell strain and culture conditions. 14
  - Radioactive labelling of cellular DNA. 14
  - UV irradiation and subsequent incubation of cell cultures. 14
  - DNA extraction and preliminary digestion. 15
  - Dialysis of bases, nucleosides and small oligonucleotides. 15
  - Dialysis of digested DNA and subsequent digestion. 15
  - Isolation of excision fragments. 16
  - Analysis of lesion-containing trinucleotides and oligonucleotides. 16
    - (1) Formic acid hydrolysis. 16
    - (2) Enzymatic hydrolysis. 17
    - (3) Alkali treatment. 17
    - (4) HPLC analysis. 17
  - Characterization of d-T[p]T, d-T[p]C, dT<>dT, and dT<>dC. 17
  - Analysis of the solubility of the small oligonucleotide, $p(dT)_{10}$, in 5% TCA. 18
  - Strong anion exchange (SAX) chromatography. 18
  - Polyacrylamide gel electrophoresis. 19

- RESULTS 20
  - Use of dialysis to remove mononucleosides from digested DNA. 20
  - Formic acid hydrolysis of UV-treated genomic DNA or excision fragments collected from post-UV incubated normal human fibroblasts. 20
  - Separation of SVP/CAP/NP1 digested unrepaired UV lesion-containing DNA into distinct species by HPLC chromatography and identification of (6-4) photoproducts. 21
  - Separation of SVP/CAP/NP1 digested excision fragments into distinct species by HPLC chromatography and identification of modified cyclobutane dimers. 22
  - Removal of photoproducts from genomic DNA of post-UV incubated normal human fibroblasts. 24
  - Accumulation of photoproducts in excision fragments from post-UV incubated normal human fibroblasts. 25
  - Kinetics of UV photoproduct repair in normal human fibroblasts. 26

State of the interpyrimidine phosphodiester bond in a (6-4) photoproduct. 27
Length of excision fragments found in post-UV incubated normal human fibroblasts. 27

DISCUSSION 29

CHAPTER THREE - REPAIR OF UV PHOTOPRODUCTS IN CHINESE HAMSTER OVARY CELLS: AN EXPLANATION FOR THEIR HIGH UV SURVIVAL . . . 58

INTRODUCTION 58

EXPERIMENTAL PROCEDURES 60
Cell strains. 60
Material, culture conditions and radioactive labelling of cellular DNA. . . . 60
UV irradiation, subsequent incubation of cell cultures, and isolation of trinucleotides derived from enzymatic digestion of genomic DNA. 60
Isolation and SAX analysis of excision fragments. 60
Analysis of lesion-containing oligonucleotides. 61

RESULTS 62
Relative sensitivity of CHO and human cells to [^{3}H]-thymidine labelling. 62
Recovery of TCA-soluble material from normal CHO and human cells. . . 62
Analysis of the TCA-soluble material by SAX-HPLC. 62
Measurement of cyclobutane dimers in excision fragments from CHO and human cells. 63
Measurement of (6-4) photoproducts and modified cyclobutane dimers in excision fragments from CHO cells. 64
Disappearance of UV Photoproducts from Genomic DNA. 65
Time course for (6-4) photoproduct and cyclobutane dimer repair in CHO and human cells. 66
Time course for unmodified versus modified cyclobutane dimers in CHO and human cells. 67
Repair of UV lesions in two UV sensitive CHO mutants. 67

DISCUSSION 68

CHAPTER FOUR - XERODERMA PIGMENTOSUM CELLS HAVE DIFFERENT ABILITIES TO REPAIR PYRIMIDINE-PYRIMIDONE (6-4) PHOTOPRODUCTS VERSUS CYCLOBUTANE PYRIMIDINE DIMERS 84

INTRODUCTION 84

EXPERIMENTAL PROCEDURES 86
Cell and culture conditions. 86

Other material and methods. 86

RESULTS 87
Measurement of UV lesions in excision fragments from XP fibroblasts. . . 87
Modification of cyclobutane dimers found in excision fragments from XP cells. 88

DISCUSSION 89

CHAPTER FIVE - THE REPAIR OF UV PHOTOPRODUCTS IN CULTURED FIBROBLASTS DERIVED FROM TRICHOTHIODYSTROPHY PATIENTS AND OTHER UV-SENSITIVE HUMAN SUBJECTS. 99

INTRODUCTION 99

EXPERIMENTAL PROCEDURES 101
Cell and culture conditions. 101
Other material and methods. 101

RESULTS 102

DISCUSSION 104

CHAPTER SIX - THE REPAIR OF A NOVEL UV-INDUCED DNA LESION, THE TA* PHOTOPRODUCT, IN NORMAL AND XP HUMAN FIBROBLASTS 114

INTRODUCTION 114

EXPERIMENTAL PROCEDURES 116
Identification of the TA* photoproduct. 116
Cell strains and culture conditions. 116
Subsequent material and methods. 116
Purification of trinucleotides by DEAE-cellulose and nuclease P1 digestion. 117
Measurement of the rate of induction of TA* lesions in genomic DNA. . . 117

RESULTS 118
Identification of the TA* photoproduct. 118
The rate of induction of TA* lesions by 254-nm light in genomic DNA. 118
Repair of TA* photoproducts in normal human fibroblasts (GM38). 119
Repair of TA* photoproducts in fibroblasts representing the various XP complementation groups. 120
TA* photoproducts and (6-4) photoproducts are repaired with very similar kinetics. 121

DISCUSSION 122

CHAPTER SEVEN - *ESCHERICHIA COLI* ALSO HAS INTRADIMER PHOSPHODIESTERASE ACTIVITY 131

INTRODUCTION 131

EXPERIMENTAL PROCEDURES 133
  Cell strains. 133
  Use of excision fragments to investigate cyclobutane dimer modification. 133
    Culture conditions and radioactive labelling of cellular DNA. . . . 133
    UV irradiation and subsequent incubation of bacterial cultures. . . 134
    Collection of excision fragments from post-UV incubated cultures. 134
    Measurement of lesions present in excision fragments from post-UV incubated cells, and assessment of the state of the intradimer phosphodiester bond. 134
  Assessment of UV lesions remaining in cellular DNA following post-UV incubation. 135
    Isolation of genomic DNA from post-UV incubated cells. 135
    Analysis of the lesions present in genomic DNA isolated from post-UV incubated cells. 135

RESULTS 136
  UV-photoproduct repair in wild-type *E. coli*. 136
  Photoproduct removal in various *E. coli* mutants. 137
  Accumulation of modified cyclobutane dimers in the DNA of excision-deficient mutants. 138

DISCUSSION 140

CHAPTER EIGHT - THE DEVELOPMENT OF AN *IN VITRO / IN VIVO* SYSTEM TO ASCERTAIN THE CELLULAR FUNCTION OF INTRADIMER PHOSPHODIESTERASE ACTIVITY. 158

INTRODUCTION 158

EXPERIMENTAL PROCEDURES 162
  Materials. 162
  Substrate construction. 162
    Preparation of pGEM vector sequences. 162
    Oligonucleotides inserted into the pGEM vector. 164
    Production and purification of cyclobutane dimer-containing AGA-6. 164
    Isolation of oligonucleotides with a modified cyclobutane dimer. 166
    Ligation of oligonucleotides into the pGEM vector and subsequent purification. 168

Assessment of the activity of DenV on unaltered and modified cyclobutane dimers. 170
DenV hydrolysis of UV-irradiated CCC plasmid DNA. 170
DenV hydrolysis of pGEM-7Zf(-) containing at a defined site (i) no lesion, (ii) an intact cyclobutane dimer, or (iii) a cyclobutane dimer with a severed intradimer phosphodiester backbone. 171
Assessment of the *in vitro* activity of *E. coli* DNA polymerase I on unmodified and modified cyclobutane dimers. 172
Substrate preparation. 172
Annealing of the reverse sequencing primer to the substrate. 172
In vitro replication. 172
Assessment of the *in vitro* activity of SP6 RNA polymerase on unaltered and modified cyclobutane dimers. 173
Substrate preparation. 173
In vitro transcription using SP6 RNA polymerase. 173

RESULTS 174
Choice of substrate and rationale of plasmid design. 174
Isolation of pGEM-7Zf(-) vector sequences. 175
Production and isolation of cyclobutane dimer-containing oligomer AGA-6. 176
Human IDP treatment of T^T-containing AGA-6. 177
Formation of a modified cyclobutane dimer-containing oligonucleotide by the photoligation of AGA-9 and AGA-8. 178
Ligation of the various oligomers into the multiple cloning site of pGEM-7Zf(-) and subsequent purification. 178
Assessment of an active DenV preparation. 179
The action of the UV endonuclease on cyclobutane dimers with or without a severed intradimer phosphodiester bond. 180
DNA synthesis by *E. coli* DNA polymerase I on a substrate containing a cyclobutane dimer with or without a severed intradimer phosphodiester bond. 181
Transcription by SP6 RNA polymerase on a substrate containing an intact or modified cyclobutane dimer. 182
DISCUSSION 183

CHAPTER NINE - SUMMARY, GENERAL CONCLUSIONS and FUTURE DIRECTIONS 221

BIBLIOGRAPHY 232

APPENDICES 253

APPENDIX A - DETAILED METHODOLOGIES UTILIZED IN IDENTIFICATION AND QUANTITATION OF UV PHOTOPRODUCTS INDUCED IN AND EXCISED FROM THE GENOMIC DNA OF LIVING CELLS 254
APPENDIX B - DETAILED METHODOLOGIES UTILIZED IN THE DEVELOPMENT OF AND *IN VITRO / IN VIVO* SYSTEM TO ASCERTAIN THE CELLULAR FUNCTION OF INTRADIMER PHOSPHODIESTERASE 278

LIST OF TABLES

TABLE I Radioactivity retained after dialysis of hydrolysed UV-irradiated (40 J/m^2) human (GM38) genomic DNA. 41

TABLE II Percentage of radioactivity in lesion peaks after nuclease P1 digestion and HPLC analysis of labelled trinucleotides (generated by enzymatic digestion of genomic DNA and enriched by dilaysis) derived from post-UV (40 J/m^2) incubated normal human fibroblasts. 42

TABLE III UV photoproducts remaining in genomic DNA of a single cell following post-UV (40 J/m^2) incubation of normal human fibroblasts. 43

TABLE IV Distribution of hydrolysed excision fragments from post-UV incubated (40 J/m^2) normal human fibroblasts into various species separated by reverse-phase HPLC. 44

TABLE V The accumulation of UV lesion-containing excision fragments in post-UV (40 J/m^2) incubated normal human fibroblasts (GM38). 45

TABLE VI Solubility of radiolabelled oligonucleotides in conditions used to isolate excision fragments. 46

TABLE VII Radioactivity recovered in TCA-insoluble and -soluble fractions from CHO cells (AA8) or human fibroblasts (GM38) after no treatment or 40 J/m^2 and various repair periods. 74

TABLE VIII Percentage of total radiolabel in major peaks after formic acid hydrolysis of TCA-soluble fractions isolated from post-UV incubated AA8 cells. 75

TABLE IX Percentage of total radiolabel in major peaks after enzymatic or alkaline hydrolysis of TCA-soluble material from post-UV incubated AA8. 76

TABLE X Radioactivity retained after dialysis of enzymatically hydrolysed UV-irradiated (40 J/m^2) AA8 genomic DNA and contained in the various photolesion peaks after subsequent nuclease P1 digestion. 77

TABLE XI UV photoproducts remaining in genomic DNA or recovered in excision fragments for post-UV (40 J/m^2) incubated normal CHO (AA8) and human (GM38) cells. 78

TABLE XII Correlation between observed and expected UDS calculated using the enzymatic digestion/ HPLC assay. 96

TABLE XIII The removal of TA* lesions from genomic DNA in normal (GM38) and XP-variant (CRL1166) human fibroblasts. 125

TABLE XIV Accumulation of UV lesions in excision fragments isolated from post-UV

(40 J/m^2) incubated and sham-irradiated *E. coli* (AB1157). 147

TABLE XV Distribution of intact and modified cyclobutane dimers contained in excision fragments isolated from post-UV (40 J/m^2) incubated and sham-irradiated *E. coli* (AB1157). 148

TABLE XVI Accumulation of UV lesions in excision fragments from post-UV (40 J/m^2) incubated *E. coli* strains characterized by different genetic backgrounds. 149

TABLE XVII Distribution of intact and modified cyclobutane dimers contained in excision fragments isolated from various post-UV (40 J/m^2) incubated (2 hr) *E. coli* strains. 150

TABLE XVIII Percentage of radioactivity representing the various classes of lesions in genomic DNA isolated from post-UV (40 J/m^2) incubated and sham-irradiated excision-deficient strains of *E. coli*. 151

TABLE XIX UV photoproducts remaining in genomic DNA isolated from two repair-deficient post-UV (40 J/m^2) incubated *E. coli* strains. 152

TABLE XX Distribution of intact and modified cyclobutane dimers contained in the genomic DNA isolated from two repair-deficient post-UV (40 J/m^2) incubated *E. coli* strains. 153

TABLE XXI A comparison of the number of cyclobutane dimers which are modified and contained within the genomic DNA of the excision-deficient strain, *uvrA*, to the number found in excision fragments of a wild-type strain. . . 154

TABLE XXII OD_{260} reading and amount of DNA in the various fractions of *Nsi*I/*Sph*I-digested pGEM-7Zf(-) eluted from the Sepharose CL-6B column. 193

TABLE XXIII Tabulation of the quantities involved and the efficiency of reactions for the procedures involving (i) isolation of the 2917-bp pGEM-7Zf(-) fragments and (ii) isolation of cyclobutane dimer and modified dimer-containing oligonucleotides. 194

TABLE XXIV Densitometric scan, of the ligation reactions shown in Figure 45, to quantitate amount of resultant substrate formed. 195

TABLE XXV Quantities of components utilized and efficiency of reaction in ligating oligonucleotides into the multiple cloning site of pGEM-7Zf(-). 196

TABLE XXVI Representative densitometric scan of relevant bands generated upon DenV hydrolysis of *Pvu*II cut [^{32}P] end-labelled pGEM-derived constructs. DNA bands were detected by polyacrylamide gel electrophoresis as illustrated in Figure 49. 197

LIST OF FIGURES

FIGURE 1 Illustration depicting the enzymatic hydrolysis / HPLC assay for quantitating UV photoproducts in [^{3}H] thymidine-labelled DNA. 47

FIGURE 2 Retention, in dialysis tubing, of small nucleic acid species as a function of dialysis time. 48

FIGURE 3 Reverse-phase HPLC radioactivity profiles of the material eluting at fractions 28-34 from reverse-phase HPLC after enzymatic digestion of [^{3}H] thymidine-labelled UV-treated DNA. 49

FIGURE 4 Reverse-phase HPLC radioactivity profiles of the material eluting at fractions 34-37 on reverse-phase HPLC after enzymatic digestion of [^{3}H] thymidine-labelled UV-treated DNA. 50

FIGURE 5 Reverse-phase HPLC radioactivity profiles of partially purified trinucleotides obtained from UV-treated (40 J/m^2) normal human fibroblast (GM38) DNA. 51

FIGURE 6 Reverse-phase HPLC radioactivity profiles of excision fragments isolated from post-UV (40 J/m^2) incubated (24 h) normal human fibroblasts (GM38). 52

FIGURE 7 Repair of cyclobutyl dimers, following 40 J/m^2 of 254-nm light, in normal human fibroblasts (GM38). 53

FIGURE 8 Repair of T[p]Py (6-4) photoproducts, following 40 J/m^2 of 254-nm light, in normal human fibroblasts (GM38). 54

FIGURE 9 HPLC radioactivity profiles of excision fragments isolated from post-UV (40 J/m^2) incubated (24 hr) normal human fibroblasts (GM38). 55

FIGURE 10 SAX profile of excision fragments isolated from post-UV (40 J/m^2) incubated (12 hr) normal human fibroblasts (GM38). 56

FIGURE 11 Polyacrylamide gel illustrating the lengths of oligonucleotides contained in the TCA-soluble fraction derived from post-UV (40 J/m^2) incubated (12 hr) normal human fibroblasts (GM38). 57

FIGURE 12 SAX-HPLC radioactivity profiles of TCA-soluble material from CHO (AA8) and human (GM38) cells. 79

FIGURE 13 Reverse-phase HPLC radioactivity profiles of TCA-soluble material from CHO (AA8) and human (GM38) cells after formic acid hydrolysis. 80

FIGURE 14 Reverse-phase HPLC radioactivity profiles of TCA-soluble material from CHO (AA8) and human (GM38) cells after enzymatic hydrolysis. 81

FIGURE 15 Repair of (6-4) photoproducts in CHO cells (AA8) and human fibroblasts (GM38) following 40 J/m^2 of 254-nm light. 82

FIGURE 16 Repair of cyclobutane dimers in CHO cells (AA8) and human fibroblasts (GM38) following 40 J/m^2 of 254-nm light. 83

FIGURE 17 Repair of cyclobutane dimers in human (normal and various XP) cell strains following 40 J/m^2 of 254 nm light. 97

FIGURE 18 Repair of (6-4) photoproducts in human (normal and various XP) cell strains following 40 J/m^2 of 254 nm light. 98

FIGURE 19 Repair of cyclobutane dimers, following exposure to 40 J/m^2 of 254-nm light, in three trichothiodystrophy cell strains and one normal human (GM38) strain. 110

FIGURE 20 Repair of (6-4) photoproducts, following 40 J/m^2 of 254-nm light, in three trichothiodystrophy cell strains and one normal human (GM38) strain. 111

FIGURE 21 Repair of cyclobutane dimers in two human cell strains, BD-1 and CVT, derived from patients displaying UV sensitivity. 112

FIGURE 22 Repair of (6-4) photoproducts in two human cell strains, BD-1 and CVT, derived from patients displaying UV sensitivity. 113

FIGURE 23 Reverse-phase HPLC chromatograms identifying the TA* photoproduct. . 126

FIGURE 24 The rate of induction of cyclobutane dimers, (6-4) photoproducts and TA* lesions in genomic DNA as a function of 254 nm-light treatment. 127

FIGURE 25 Reverse-phase HPLC chromatogram of nuclease P1-digested lesion-containing trinucleotides isolated from the genomic DNA of UV (40 J/m^2; 254-nm light) irradiated normal human fibroblasts (GM38). 128

FIGURE 26 Repair of cyclobutane dimers, (6-4) photoproducts, and TA* lesions, following exposure of normal human fibroblasts to 40 J/m^2 of 254-nm light 129

FIGURE 27 Repair of (6-4) photoproducts and TA* lesions in normal and various XP human fibroblasts following exposure to 40 J/m^2 of 254-nm light. 130

FIGURE 28 Reverse-phase HPLC radioactivity profiles of excision fragments isolated from post-UV (40 J/m^2) incubated (2 hr) wild-type *E. coli* (AB1157) cells. 155

FIGURE 29 Repair of cyclobutyl dimers and (6-4) photoproducts, following 40 J/m^2 of 254-nm light, in wild-type *E. coli* (AB1157). 156

FIGURE 30 Accumulation of intact and modified cyclobutane dimers in excision fragments from post-UV (40 J/m^2) incubated wild-type *E. coli* (AB1157). 157

FIGURE 31 Assay system used by Mellon and Hanawalt (1989) to detect repair of cyclobutane dimers in specific regions of the genome (in this example for *E. coli*). 199

FIGURE 32 The structure and relevant sequences of the pGEM-7Zf(-) vector (Promega). 200

FIGURE 33 Synthetic oligonucleotides used, in conjunction with pGEM-7Zf(-), to construct a double-stranded closed circular substrate with (i) no lesion, (ii) a cyclobutane dimer at a specific TT sequence, and (iii) a modified dimer at the same position. 201

FIGURE 34 Agarose gel of pGEM-7Zf(-) before and after cutting with restriction enzymes. 202

FIGURE 35 Agarose gel showing ligation products of reactions involving linearized pGEM-7Zf(-). 203

FIGURE 36 HPLC UV profile illustrating the separation of the T^T-containing AGA-6 from its parent molecule, by capped reverse-phase chromatography. 204

FIGURE 37 Reverse-phase HPLC UV profile displaying **A**: T^T-containing AGA-6; **B**: after treatment with photolyase; **C**: after photochemical reversal. . . . 205

FIGURE 38 HPLC UV profile illustrating the separation of 5'-phosphorylated T^T-containing AGA-6 from the unphosphorylated molecule, by capped reverse-phase chromatography. 206

FIGURE 39 Reverse-phase HPLC chromatogram displaying the inactivity of IDP on T^T-containing AGA-6. 207

FIGURE 40 Polyacrylamide gel illustrating the inactivity of IDP on T^T-containing AGA-6. 208

FIGURE 41 Reverse-phase HPLC chromatogram representing the isolation of a modified cyclobutane dimer-containing oligonucleotide generated by photoligating the two smaller oligomers, AGA-9 and AGA-8. 209

FIGURE 42 Reverse-phase HPLC chromatogram displaying **A**: photoligated oligonucleotides; **B**: after monomerization by photochemical reversal. . . 210

FIGURE 43 HPLC chromatogram illustrating the separation of the 5'-phosphorylated modified dimer-containing oligonucleotide from the unphosphorylated molecule, by capped reverse-phase chromatography. 211

FIGURE 44 Hybridized oligonucleotides, displaying the non-complementary ends. . . . 212

FIGURE 45 Agarose gel depicting the closed circular plasmid DNA generated by ligating oligonucleotides to the 2917-bp fragment of the pGEM-7Zf(-) vector. 213

FIGURE 46 Low melt agarose gel, run in the presence of ethidium bromide, illustrating the products of a large scale ligation reaction involving T^T-containing AGA-6, AGA-4, AGA-7, and the 2917-bp *NsiI*/*SphI* fragment of pGEM-7Zf(-). 214

FIGURE 47 Illustration of site-specific cyclobutane dimer-containing substrate generated by inserting a dimer-containing oligonucleotide into the multiple cloning site of the pGEM-7Zf(-) vector. 215

FIGURE 48 Detailed representation of sequences relevant to *in vitro* replication and transcription in constructed substrate. 216

FIGURE 49 Agarose gel displaying the activity of 3 independent DenV preparations on irradiated and unirradiated plasmid DNA. 217

FIGURE 50 Polyacrylamide gel displaying the activity of T4 UV endonuclease (DenV) on the pGEM-derived constructs. 218

FIGURE 51 Polyacrylamide denaturing gel illustrating *in vitro* replication of the pGEM-derived constructs by *E. coli* DNA polymerase I. 219

FIGURE 52 Polyacrylamide denaturing gel illustrating *in vitro* transcription of the pGEM-derived constructs with SP6 RNA polymerase. 220

ABBREVIATIONS.

Amp^R	ampicillin resistance
ATP	adenosine-5'-triphosphate
bp	base pair(s)
bromophenol blue	3',3'',5',5''-tetrabromophenolsulfonaphthalein
c-AMP	cyclic adenosine monophosphate
C^C	all cytidine-cytidine cyclobutane dimers irrespective of molecular configuration
C[p]C	cytidine-cytidine (6-4) photoproduct
CAP	calf alkaline phosphatase
CCC	covalently closed circular
CHO	Chinese hamster ovary
Ci	Curie
cpm	counts per minute
CS	Cockayne syndrome
CTP	cytidine-5'-triphosphate
d-ApT{p}A	TA* photoproduct-containing trinucleotide derived from SVP digestion of the copolymer d(AT)$_n$
d-T{p}A	thymidylyl-(3'»5')-adenosine containing a TA* photoproduct and generated by enzymatic digestion of UV-treated DNA
d-T<p>C	thymidylyl-(3'»5')-cytidine containing a cyclobutane dimer and generated by enzymatic digestion of UV-treated DNA
d-T[p]C	thymidylyl-(3'»5')-cytidine containing a (6-4) photoproduct and generated by enzymatic digestion of UV-treated DNA
d-T[p]Py	includes both d-T[p]C and d-T[p]T

d-T<p>T	thymidylyl-(3'»5')-thymidine containing a cyclobutane dimer and generated by enzymatic digestion of UV-treated DNA
d-T[p]T	thymidylyl-(3'»5')-thymidine containing a (6-4) photoproduct and generated by enzymatic digestion of UV-treated DNA
d-TpT<>dT	modified dimer-containing thymidylate
d-TpT<p>T	cyclobutane dimer-containing thymidylate
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddH$_2$O	double-distilled water
DEAE	diethylaminoethyl
DHFR	dihydrofolate reductase
dGTP	2'-deoxyguanosine-5'-triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dpm	disintegrations per minute
dT	thymidine
dT<>dC	cyclobutane thymidine-cytidine (modified) dimer and generated by enzymatic digestion of UV-treated DNA
dT<>dT	cyclobutane thymidine-thymidine (modified) dimer resulting from SVP/CAP/NP1 digestion of UV-treated DNA
dTTP	2'-deoxythymidine-5'-triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid, disodium salt
EMS	ethyl methanesulfonate
g	gram(s)
GTP	guanosine-5'-triphosphate

HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
hr	hour(s)
i.d.	inner diameter
ID-pGEM	cyclobutane dimer-containing derivative of pGEM-7Zf(-) (with lesion at position 32-33)
IDP	intracyclobutyl pyrimidine dimer-DNA phosphodiesterase
in.	inch
IPTG	isopropyl-β-D-thiogalactoside
kDa	kilodalton(s)
l	litre(s)
LB	Luria broth
lb.	pound(s)
M	molar
mol	mole(s)
MW	molecular weight
m-pGEM	pGEM-7Zf(-) modified by insertion of oligonucleotides into the multiple cloning site
MD-pGEM	modified dimer-containing derivative of pGEM-7Zf(-) (with lesion at position 32-33)
N	normal
NK	natural killer
NP1	nuclease P1
nt	nucleotide(s)
OD	optical density
Py^Py	includes all pyrimidine cyclobutane dimers irrespective of molecular configuration

Py[p]Py	includes T[p]C, T[p]T, and C[p]C
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SAX	strong anion exchange
SDS	sodium dodecyl sulfate
sq.	square
SVP	snake venom phosphodiesterase
TA*	thymidine dimerized to an adjacent adenine
T^C	includes all thymidine-cytidine and cytidine-thymidine cyclobutane dimers irrespective of molecular configuration
T^T	includes all thymidine-thymidine cyclobutane dimers irrespective of molecular configuration
T<>T	cyclobutane thymine-thymine dimers seen after formic acid hydrolysis
T<>U	cyclobutane uracil-thymine dimer seen after formic acid hydrolysis (includes U<>T)
T[ ]Py	alkali-modified (6-4) photoproduct
T[p]C	thymidine-cytidine (6-4) photoproduct
T[p]Py	includes T[p]C and T[p]T
T[p]T	thymidine-thymidine (6-4) photoproduct
TCA	trichloroacetic acid
TDP	thymidine diphosphate
TEMED	*NNN'N'*-tetramethylethylene-diamide

Thy	thymine
TMP	thymidine monophosphate
Tris	Tris(hydroxymethyl)aminomethane
ts	temperature sensitive
TTD	trichothiodystrophy
TTP	thymidine-5'-triphosphate
UDS	unscheduled DNA synthesis
UV	ultraviolet
v/v	volume per volume
V	volts
W	watts
w/v	weight per volume
XP	xeroderma pigmentosum

CHAPTER ONE - AN OVERVIEW

In the past ten years, largely due to the tremendous growth in the technologies available to molecular biology, our understanding of the ways in which cells cope with ultraviolet (UV)-induced DNA damage has progressed immensely. From a good general knowledge of nucleotide excision repair, combined with a basic understanding of damage recognition / DNA incision in *Escherichia coli*, we have come to learn the specific function of each component of the UvrABC nuclease, as well as the interaction of the members within the "repairosome" complex and with the damaged DNA substrate (for review, see Grossman and Yeung, 1990; Van Houten, 1990). It is now appreciated that only UvrA has a capacity to bind duplex DNA. It binds ATP, which in turn, allows UvrA to form a homodimer and bind productively to DNA (Oh *et al.*, 1989). It is believed that UvrA acts to deliver UvrB to the site of damage. UvrB interacts with UvrA as a $(UvrA)_2(UvrB)_1$ complex. The ATPase activity of UvrB, apparent only after interaction with UvrA, enables topological unwinding of the DNA, and the resulting translocation of the complex to a site of DNA damage (Grossman and Yeung, 1990). It is then believed that UvrA dissociates, and it is the binding of UvrC to the UvrB-DNA damage structure which produces a functional incision complex (Orren and Sancar, 1989; Seeley and Grossman, 1990). Damage excision is then achieved by a dual incision event, 7 nucleotides 5' to the site of damage, and 3 - 4 nucleotides 3' (Yeung *et al.*, 1983; Sancar and Rupp, 1983). The release of the resulting oligonucleotide requires the helicase activity of the *uvrD* gene product (Caron *et al.*, 1985). It is intriguing that, although the *uvrA*, *uvrB*, and *uvrC* mutants and their cognate genes were each discovered because of and named for their UV-sensitive phenotype, a cyclobutane dimer is, at least *in vitro*, a relatively poor substrate for the UvrABC endonuclease. This general incision complex recognizes a large array of bulky lesions occurring in DNA (Sancar and Rupp, 1983; Sancar *et al.*, 1985; Van Houten *et al.*, 1986; Grossman *et al.*, 1988; Lambert *et al.*, 1989). However, it has been recently found that, *in vivo*, photolyase actually stimulates cyclobutane dimer repair, by pre-binding to these lesions (Sancar *et al.*, 1984; Sancar and Smith, 1989).

Advancements have also been made in understanding the specific interactions of the UmuCD complex with the DNA polymerase at a blocked replication fork. The *umuC* and *umuD* genes, like the *uvr* genes, belong to a family of damage-inducible genes under the control of RecA and LexA proteins (Witkin, 1976; Little and Mount, 1982; Walker, 1984). Following induction, the *umuC* and *umuD* gene products allow replication to continue, albeit with reduced fidelity, past polymerase-blocking cyclobutane dimers (Bridges and Woodgate, 1984; Bridges and Woodgate, 1985). Although UmuC and UmuD appear to act in concert, there is as yet no evidence for a UmuC-UmuD complex within the cell. Instead, RecA protein appears to direct cleavage of UmuD (Shinagawa *et al.*, 1988), and it is the truncated product, UmuD′, which binds tightly to UmuC (Woodgate *et al.*, 1989). Although the total picture has yet to be completed, it is now recognized that RecA is not only necessary for the expression of these proteins and the cleavage of UmuD. It is also, itself, an integral part of mutagenic bypass, interacting with the UmuC-UmuD′ complex and polymerase III at the replication fork (Bridges and Woodgate, 1985; Lu *et al.*, 1986; Lu and Echols, 1987; Nohmi *et al.*, 1988; Woodgate *et al.*, 1989).

In addition to mutagenic bypass, RecA has emerged as an essential component in other facets of the SOS response. It participates directly in recombinational bypass (Livneh and Lehmann, 1982), and is indispensable for recovery from inhibition of DNA synthesis following UV irradiation, in a process distinct from mutagenic bypass (Khidhir *et al.*, 1985).

A detailed comprehension of the events occurring in a relatively simple system such as *E. coli* is essential to tackling those arising in the more complex eukaryotic cell. Given our current understanding of many intricate details of nucleotide excision repair and mutagenic bypass in *E. coli*, the rapid growth in our understanding of the management of UV-induced DNA damage in eukaryotic cells is not surprising. Many of the proteins and cognate genes responsible for nucleotide excision repair in *Saccharomyces cerevisiae* and *Saccharomyces pombe* have been extensively studied and are now well understood (Haynes and Kunz, 1981; Sung *et al.*, 1987a; Friedberg, 1988; Broughton *et al.*, 1991; Lehmann *et al.*, 1991). Although UV sensitive mutants in mammalian cells have been identified for quite some time [eight complementation groups of the genetic disease, xeroderma pigmentosum (XP) (Cleaver and Bootsma, 1975) in humans, and several non XP-compensating complementation groups of artificially derived rodent lines (Thompson *et al.*, 1981; Thompson *et al.*, 1985; Thompson *et al.*, 1987)], our knowledge until recently, has been quite limited, mainly due to the difficulty associated with manipulating mammalian systems and cloning mammalian genes (Lehmann, 1985; Schultz *et al.*, 1985; Gebara *et al.*, 1987; Mayne *et al.*, 1988). Notwithstanding the recent advancements in our knowledge of mammalian excision repair, as will be clear from the discussion below, this field is still in the a of transition.

Seven of the eight complementation groups of XP have been known for some time to harbor a defect in the initial incision step of nucleotide excision repair (Kraemer, 1983; Wood *et al.*, 1988). This minimum of seven genes associated with damage recognition / DNA incision plus an additional eight suggested by isolation of UV-sensitive rodent mutants with unrelated incision defects, as compared to the three "repairosome" genes in *E. coli* (*uvrA*, *uvrB*, and *uvrC*), reinforces the complexity of mammalian systems. As a result, seemingly little progress was made past this stage for quite some time. The isolation of UV-sensitive Chinese hamster ovary (CHO) cell lines (Thompson *et al.*, 1981; Thompson *et al.*, 1985) has allowed the cloning of several human repair genes. This has been accomplished by introducing human DNA into a particular rodent cell line, often by cell fusion or DNA mediated gene transfer. Continued culturing causes loss of a large proportion of the human sequences, and if selection for the UV-resistant phenotype is maintained, the complementing human sequence is present and can be identified by various means. Each human repair gene, isolated in this way, only compensates for the defect in members of the UV-sensitive CHO complementation group in which it was cloned (for review, see Rubin, 1988). Although, as assessed by cell fusion, the defect in any of the various UV-sensitive CHO cell lines does not correspond to the defect in any XP complementation group, two of the five human repair genes cloned by their exploitation have proved to be XP-complementing genes. The first such gene to be cloned, *ERCC-1* (Rubin *et al.*, 1983; Westerveld *et al.*, 1984; Rubin *et al.*, 1985), complements the defect in group 1 UV-sensitive rodent cells (van Duin *et al.*, 1988), but not any of the eight XP complementation groups (van Duin *et al.*, 1989). It displays considerable homology to *rad10* (a bulky-lesion repair gene and a member of the *rad10* epistasis group of *Saccharomyces cerevisiae*) including conservation of a 3' antisense transcription unit (van Duin *et al.*, 1989). It also has limited homology to *uvrA* and *uvrC*, and contains a putative DNA binding site (Doolittle *et al.*, 1986; Hoeijmakers *et al.*, 1986; van Duin *et al.*, 1986). *ERCC-2* complements group 2 rodent cells (Regan *et al.*, 1990) and shows strong homology (73%) to *rad3* of yeast (Weber *et al.*, 1988; Weber *et al.*, 1990), and by inference of this homology, may have helicase activity (Sung *et al.*, 1987a; Sung *et al.*, 1987b; Harosh *et al.*, 1989). *ERCC-3* (Weeda *et al.*, 1990a), *ERCC-5* (Cox and Gedde-Dahl, 1985; Thompson *et al.*, 1987b) and *ERCC-6* (Hoeijmakers *et al.*, 1989; Troelstra *et al.*, 1990) have also been cloned recently, and the chromosomal location of *ERCC-4* (Siciliano *et al.*, 1988; Smith and Simpson, 1989) has been identified. Each complements the corresponding UV-sensitive rodent group (i.e. 3, 5, 6, and 4, respectively). *ERCC-1* and *ERCC-3* are now believed to be part of the cellular machinery which recognizes and incises damaged DNA. This speculation is based on *in vitro* excision repair studies which have demonstrated a requirement for their gene products under conditions where chromatin structure is lacking (eliminating chromatin disassembly as a potential function for these proteins), and in the absence of replication and transcription (thus relinquishing an obligatory interaction of the *ERCC-1* and *ERCC-3* gene products with these processes) (Biggerstaff and Wood, 1992). Information on the involvement of the other *ERCC* genes in human nucleotide excision repair is rapidly accumulating.

In another approach, putative human repair genes have been cloned by virtue of their functional homology to known yeast repair genes (Koken *et al.*, 1991). Further tactics to uncover the associated proteins are underway (Hoeijmakers *et al.*, 1990).

Progress has recently been made in the cloning of various XP genes. A human DNA sequence has been isolated which fully complements XP group A cells (Tanaka *et al.*, 1989; Tanaka *et al.*, 1990; Ishizaki *et al.*, 1990; Miura *et al.*, 1991). The specific protein species which complements XP group A cells has strong affinity for damaged DNA, being 10^3-fold higher than for an undamaged template (Robins *et al.*, 1991). Although the XP-A defect does not appear to be an endonuclease deficiency per se (Tsongalis *et al.*, 1990), it is predicted to be a part of the incision complex rather than serving a regulatory role, a chromatin disassembly function, or an involvement with replication and/or transcription (Robins *et al.*, 1991). The *ERCC-3* gene corrects the repair defect in XP group B and is implicated in Cockayne's syndrome (Weeda *et al.*, 1990b; Weeda *et al.*, 1991). A cDNA has been isolated by polymerase chain reaction which partially complements the UV sensitivity of XP group C cells (Teitz *et al.*, 1990), but little more is known to date about the cognate gene product. Nonetheless, it is believed that the defect in XP group C cells affects "domain-limited" repair (Mansbridge and Hanawalt, 1983; Mullenders *et al.*, 1984; Mullenders *et al.*, 1988; van Zeeland *et al.*, 1988) associated specifically with the nuclear matrix, but this effect may simply result from reduced amounts of normal repair enzymes (Karentz and Cleaver, 1986; Cleaver, 1986; Cleaver, 1987; Kantor and Elking, 1988; Kantor *et al.*, 1990). The most current information equates the XP-D gene to *ERCC-2* (Arrand *et al.*, 1989; Tanaka *et al.*, 1990). In addition, an apparent XP group E factor has been purified and appears to be a cyclobutane dimer binding-protein, sharing partial homology with lower eukaryotic DNA photolyase (Chu and Chang, 1988).

The development of an *in vitro* nucleotide excision repair system (Wood *et al.*, 1988; Ullah *et al.*, 1989) has not only allowed isolation of proteins involved in human nucleotide excision repair (Robins *et al.*, 1991) and the study of cloned gene product (Biggerstaff and Wood, 1992), but it has aided in the identification of other factors required in the repair of damaged DNA. For example, it has been demonstrated recently that the replication protein, SSB, is an essential component of this process (Coverley *et al.*, 1991). Even more recently, the need for proliferating nuclear antigen has been described, implicating DNA polymerase δ in repair synthesis (Shivji *et al.*, 1992). An additional accomplishment, made possible by this *in vitro* human repair system, has been documentation of the incision position relative to the DNA damage (Huang *et al.*, 1992). Like the *E. coli* UvrABC complex, the human nucleotide excision nuclease incises on both sides of a thymine cyclobutane dimer. However, the oligonucleotide generated by this incision is somewhat larger, having cleaved at the 22nd phosphodiester bond 5' and the 6th phosphodiester bond 3' to this photodimer.

Another relatively recent pursuit has been aimed at understanding the differences in the way cells deal with, and the relative mutagenic and cytotoxic importance of, cyclobutane dimers versus 6-4'-(pyrimidin-2'-one)-pyrimidine photoproducts [so-called (6-4) photoproducts]. The isolation of an antibody specific for (6-4) photoproducts has allowed the development of an radioimmunoassay to monitor, for the first time, the removal of this class of UV lesions (Mitchell *et al.*, 1985). It quickly became obvious that cells repair these minor photoproducts, along with their photolysed derivatives (the Dewar pyrimidinones), much more rapidly than the more prevalent cyclobutane dimers (Mitchell *et al.*, 1985; Mitchell, 1988b). Although the repair of cyclobutane dimers in rodent cells is greatly reduced compared to that occurring in human cells, their UV survival is similar. However, (6-4) photoproduct removal is analogous in cells of these different species (Mitchell and Nairn, 1989). This led to the hypothesis that (6-4) photoproducts represent the major cytotoxic damage (Mitchell *et al.*, 1985), a premise later extended by studying the biological effects of UV light on an XP group A revertant line. Although having regained normal UV-survival and now capable of wild-type (6-4) photoproduct repair, this revertant remains unable to remove cyclobutane dimers (Cleaver *et al.*, 1988; Cleaver, 1989), indicating that cytotoxic and mutagenic repercussions of UV light are due to the introduction of (6-4) photoproducts. Although (6-4) photoproduct repair also correlates with the removal of blocks to replication (Mitchell *et al.*, 1990a), other analyses question the absolute importance of (6-4) photoproducts. Transfection with the cyclobutane dimer-specific repair gene, bacteriophage T4 *denV*, reduces the UV sensitivity of repair-deficient cell lines, indicating the cytotoxic relevance of cyclobutane dimers (Valerie *et al.*, 1985; Valerie *et al.*, 1987). In addition, mutation studies have shown that cyclobutane dimers, as well as (6-4) photoproducts, are mutagenic (Glickman *et* *al.*, 1986; Protić-Sabljić *et al.*, 1986; Glickman, 1989; Dorado *et al.*, 1991). Furthermore, studies in *E. coli* demonstrate that although (6-4) photoproducts are the more mutagenic lesion, their cytotoxicity is similar to that of cyclobutane dimers (Tang *et al.*, 1986).

Although (6-4) photoproducts have certainly gained the recognition they deserve, resolution of the apparent controversy of high UV survival given reduced overall cyclobutane dimer removal may lie in a recently discovered phenomenon not accounted for in the (6-4) photoproduct studies, namely preferential repair of transcriptionally active sequences (Bohr *et al.*, 1986; Bohr *et al.*, 1987). Moreover, it now appears from *in vivo* studies, that rapid (6-4) photoproduct repair is a characteristic of rapidly proliferating cells, and that terminal differentiation causes a reduced repair capacity in this regard (Tofilon and Meyn, 1988; Mitchell *et al.*, 1990a).

Preferential repair of cyclobutane dimers from transcriptionally active sequences was initially described in rodent cells (Bohr *et al.*, 1985), and offered as a ready explanation for the high UV survival characteristic of these cells. It was later described in human cells (Mellon *et al.*, 1986), as well as in *E. coli* (Mellon and Hanawalt, 1989). One problem with the proposal that preferential repair can account for high UV survival in the absence of general cyclobutane dimer repair is that XP group A revertant lines, which display normal UV survival and normal (6-4) photoproduct repair, are unable to remove cyclobutane dimers both globally and from actively transcribed sequences. The same scenario has been demonstrated for a CHO revertant (Mitchell *et al.*, 1990e).

Preferential repair is not only peculiar to transcriptionally active regions of the genome, but distinctive to the transcribed strand itself, and thus appears to be directly coupled to transcription (Mellon *et al.*, 1987). Transfection of a repair-deficient CHO cell line with the cloned T4 *denV* gene increases overall cyclobutane dimer removal, but it remains totally devoid of preferential repair (Bohr and Hanawalt, 1987). This, too, indicates an intimate relationship between the indigenous excision complex and the transcription machinery which cannot be duplicated by a foreign repair enzyme. Furthermore, the correlation of demethylation in specific genomic regions with accompanying enhanced cyclobutane dimer repair links preferential repair to transcription (Ho *et al.*, 1989) and offers an explanation for the dependence of UV repair on biological imprinting (Cleaver *et al.*, 1989). Preferential repair also appear to be a recovery characteristic for other bulky lesions, including aflatoxin B_1, psoralen photoadducts, and (6-4) photoproducts (although not nearly as dramatic as in the case of cyclobutane dimers for this other

UV lesion) (Leadon and Hanawalt, 1986; Vos and Hanawalt, 1987; Thomas *et al.*, 1989), but interestingly this is not a phenomenon associated with lesions repaired by AP endonucleases, such as *N*-methylpurines (Scicchitano and Hanawalt, 1989). This, then, implies that preferential repair of transcriptionally active sequences is specific to nucleotide excision repair as well as linked to transcription. However, the loss of preferential repair with *in vitro* terminal differentiation in genes which continue to be actively transcribed threatens to jeopardize this premise (Bill *et al.*, 1991). This may, however, represent an artifact of cell culturing as it has been demonstrated that mouse skin shows an early rapid mode of cyclobutane dimer repair which is lost with cell culturing (Mullaart *et al.*, 1988). Nevertheless, another recent finding goes against our perception of the role of preferential repair in UV survival. Two different parental CHO cell lines, each of which exhibits similar resistance to several DNA damaging agents including psoralen, do not both display preferential repair of psoralen adducts in the actively transcribed rRNA genes (Wauthier *et al.*, 1990). Such results make it clear that preferential repair is not fully understood.

One UV-sensitive human disorder, Cockayne syndrome (CS) (Mayne and Lehmann, 1982; Klocher *et al.*, 1985; Mayne *et al.*, 1988), has been linked to a defect in preferential repair. In addition, the product of the *mfd* (mutation frequency decline) gene in *E. coli* (George and Witkin, 1974) has been found to sequester the repair complex to transcriptionally active sequences (Selby *et al.*, 1991; Selby and Sancar, 1991).

A different but related line of study has recently questioned the direct association of a DNA repair defect in human cells with a high onset of cancer (for review, see Cleaver, 1990). Whereas XP patients are at high risk of developing neoplasia, this is not true of certain other diseases displaying UV sensitivity and DNA repair deficiencies, such as CS and trichothiodystrophy (TTD). It is now widely believed that cancer predisposition in XP patients stems not only from the repair defect, but also requires a natural killer cell deficiency, related to a catalase inadequacy (Vuillaume *et al.*, 1986; Crawford *et al.*, 1988), and the resultant defective immune surveillance. Thus two mutant genes would be required to confer the XP phenotype (Lambert and Lambert, 1985; Lehmann and Norris, 1989; Norris *et al.*, 1990; Lehmann and Bridges, 1990).

In our laboratory, recent related studies have focused on an intriguing observation made some time ago (Paterson *et al.*, 1984). The ability of any given cell strain to repair UV-induced DNA damage has classically been assessed by two methods: (i) unscheduled DNA synthesis (UDS) or non S-phase repair synthesis (Cleaver, 1968) and (ii) the disappearance of UV endonuclease-sensitive sites (Paterson *et al.*, 1981). Often the repair capacity determined by these two methods coincide, but XP groups D and F strains are notable exceptions. XP group D cells shows substantial UDS (~20-55% of normal) and yet virtually no removal of UV endonuclease-sensitive sites. XP group F cells exhibits the reverse discrepancy (Kraemer, 1983; Zelle and Lohman, 1979). This dilemma prompted members of our laboratory to look more closely at the unrepaired lesions which remain in the genome of post-UV incubated XP group D cells. A series of alkaline sucrose gradient studies, classically used to size genomic DNA after UV endonuclease treatment, had a quite unpredicted outcome. It was found that if genomic DNA isolated from post-UV incubated XP group D cells was treated with photolyase prior to analysis under denaturing conditions, the molecular weight of the DNA decreased. Since monomerization of a cyclobutane dimer does not normally alter the size of DNA and since the appearance of such sites requires post-UV incubation, this result implied that during repair in XP-D cells, an intradimer phosphodiester cleavage results in a cyclobutyl ring which bridges a single-stranded nick (Paterson *et al.*, 1984). An early hypothesis, that the previously unexplained UDS could be accounted for by repair synthesis at a free 3'OH end generated by the intradimer phosphodiester severage, was soon abandoned by the realization that the modification could also be uncovered in other XP complementation groups, notably A and C (Paterson *et al.*, 1987). Further studies demonstrated that excision fragments isolated from post-UV incubated normal human fibroblasts contain dimers with this configuration (Weinfeld *et al.*, 1986). Photochemical reversing fluences of 254-nm light induced the release of thymidine and TMP, suggesting the terminal location of cyclobutane dimers within the excision fragment, and their lack of a interpyrimidine phosphodiester bond. This, then, led to the premise of intradimer phosphodiester cleavage as a prerequisite to damage recognition and incision during nucleotide excision repair.

Since this initial discovery, research in the area of UV photoproduct repair in our laboratory has focused primarily on isolating the protein responsible for intracyclobutyl pyrimidine dimer-DNA phosphodiesterase (IDP) activity, cloning the cognate gene, and elucidating the cellular function of IDP. In conjunction with this work, the development of an assay system to readily detect IDP activity (Liuzzi and Paterson, in press) has stimulated the evolution of a new strategy to accurately monitor the removal of various UV photoproducts from DNA during post-UV incubation (see Chapter 2). This has allowed detailed assessment of the repair capacity, monitoring (6-4) photoproduct removal as well as cyclobutane dimer repair, of a number of UV-sensitive and normal cell strains, including the various XP complementation groups (Chapter 3), normal and UV-sensitive Chinese hamster cells (Chapter 4), cells derived from TTD patients (Chapter 5), and other previously uncharacterized human UV-sensitive fibroblast strains (Chapter 5). Such studies have confirmed and expanded related studies using alternate means of measuring repair (Zelle and Lohman, 1979; Paterson *et al.*, 1981; Mitchell, 1988a). Furthermore, this approach has facilitated repair analysis of a newly discovered photoproduct, the TA* lesion (Chapter 6), and has extended the identification of modified dimer sites (characterized by a cleaved intradimer phosphodiester bond; Chapters 2 through 5). As *E. coli* offers many advantages to molecular biology due to its relative genetic simplicity and years of study, this has become the model organism to further our understanding of intradimer phosphodiester cleavage as it pertains to the metabolic processing of UV-induced DNA damage (Chapters 7 and 8). Although this work is still in its infancy, evidence is beginning to accumulate which suggests that the role of IDP is in allowing the transcription complex to bypass these otherwise polymerase blocking lesions (Chapter 8).

Chapters 2 through 8 of this doctoral dissertation each represent a comprehensive investigation which has been or will be submitted for publication in a condensed form. Therefore each chapter includes background information, methodologies, results, and discussion relevant to the immediate subject of inquiry. Because the experimental procedures presented in each chapter are brief due to the length restrictions of journals, a more detailed account of the materials and methods is presented in three appendices at the end of this dissertation. In addition, the cross-referencing which now appears between chapters will, in their final paper format, reference published or submitted papers.

CHAPTER TWO - REPAIR OF CYCLOBUTANE DIMERS AND (6-4) PHOTOPRODUCTS IN NORMAL HUMAN FIBROBLASTS: USE OF A NEW METHODOLOGY TO MONITOR THE REPAIR OF UV-INDUCED DNA DAMAGE

INTRODUCTION

It has previously been demonstrated that excision fragments isolated from post-UV incubated normal human fibroblasts contain cyclobutane dimer sites with a broken intradimer phosphodiester bond (Weinfeld *et al.*, 1986). Cyclobutane dimer photoreversal-induced liberation of thymidine and thymidine monophosphate was employed as an indication of hydrolysed intradimer phosphodiester linkages. In addition, post-UV incubated cells from several complementation groups representing the human genetic disorder, xeroderma pigmentosum (XP), which themselves are deficient in UV-photoproduct repair, accumulate these modified cyclobutane dimers sites in their genomic DNA (Paterson *et al.*, 1984). This is visualized as a reduction in molecular weight of the genomic DNA, as measured on an alkaline sucrose gradient, after exhaustive treatment with photolyase. Classically, the capability of a cell to repair DNA damage has been assessed by two methodologies, (i) unscheduled DNA synthesis (UDS) (Cleaver, 1968) and (ii) removal of UV endonuclease-sensitive sites (Paterson *et al.*, 1981). UDS gauges repair synthesis in non-S phase cells. The incorporation of a radioisotope into genomic DNA during post-UV incubation can be directly visualized, enabling comparison between normal and UV-sensitive cells which have been treated in a similar manner. The second assay system measures the ability of a UV damage-specific endonuclease to incise the DNA. As damage is removed subsequent to UV irradiation, the number of such sites diminishes in a time-dependent fashion. The disappearance of these sites is compared to the same experiment in normal cells. Although for most purposes these two techniques have proven indistinguishable, they do not always give comparable results. XP groups D and F are notable exceptions. In repair, XP group D shows almost no disappearance of UV endonuclease-sensitive sites whereas UDS is substantial (20 - 55% of normal). XP group F shows the opposite discrepancy (Zelle and Lohman, 1979; Kraemer, 1983). These two methodologies, when employed alone, have several other distinct shortcomings. UDS give only an indication of the total amount of repair occurring with no specificity to lesion type, whereas most UV endonucleases (e.g., T4 DenV) incise damaged DNA only at a cyclobutane dimer site (Gordon and Haseltine, 1980), and therefore gives no information as to the repair of other UV lesions.

During nucleotide excision repair, small single-stranded lesion-containing oligonucleotides, termed excision fragments, are released from the genomic DNA and retained within the cell for at least 24 hr (La Belle and Linn, 1982; Weinfeld *et al.*, 1986). UV photoproducts located within these molecules have been conventionally quantitated by formic acid hydrolysis (Love and Friedberg, 1983). Although this is an excellent means of determining the total number of cyclobutane dimers in a given sample, this assay has two major shortcomings. Firstly, although the cyclobutyl bridge is very stable in hot acid, this harsh procedure liberates free bases, or in the case of a cyclobutane dimer, two pyrimidines linked by a cyclobutane ring (Varghese and Day, 1970). This, then, does not allow assessment of the state of the intradimer phosphodiester bond. Secondly, another UV lesion, the (6-4) photoproduct, which has been regarded by many as the biologically important lesion (Cleaver *et al.*, 1988), is not stable under these conditions and therefore cannot be quantitated (Wang and Varghese, 1967).

Radioimmunoassays have recently been used to successfully and independently monitor the removal of cyclobutane dimers and (6-4) photoproducts (Mitchell *et al.*, 1982; Mitchell *et al.*, 1985; Mitchell *et al.*, 1987; Cleaver *et al.*, 1987; Mitchell and Nairn 1987; Karentz *et al.*, 1987; Mori *et al.*, 1990). Unfortunately, however, antibodies do not always give definite results, since any conformational change in the antigen can render it refractory to antibody recognition. As an example, cyclobutane dimer removal, when measured by a radioimmune assay, appears to be slightly, but significantly, faster than when measured by conventional methods (Williams and Cleaver, 1979; Mitchell *et al.*, 1982; Vijg *et al.*, 1984; Mitchell *et al.*, 1985). This is likely due to some modification in the dimer prior to excision which destroys antibody recognition, thus making it appear as though the dimer has been removed (Paterson *et al.*, 1984; Mitchell *et al.*, 1987; Roth *et al.*, 1987; Liuzzi *et al.*, 1989). Although it has been demonstrated that the rapid (6-4) photoproduct excision, as monitored by radioimmunoassay, is not due to conversion to the Dewar isomer (Mitchell *et al.*, 1990a), this does not exclude conformational change as a potential weakness of an assay based on antibody recognition.

A new procedure was deemed essential which would allow more precise characterization and quantitation of UV-induced lesions present in both genomic DNA and excision fragments. The development of this new assay system was inspired largely by the ongoing studies of Liuzzi *et al.* (1989) designed to measure the action of several exonucleases (some of which also have endonuclease activity) on cyclobutane dimer-containing trithymidylates.

Calf spleen phosphodiesterase, mung bean nuclease, and nuclease S1 can all be shown to cleave the 5' internucleotide bond from the 3'-cyclobutane dimer-containing trithymidylate isomer (d-TpT<p>T), but not cleave the 3'-nucleotide from the 5'-isomer. Alternatively, snake venom phosphodiesterase cleaves the 3'-nucleotide from the 5'-isomer (d-T<p>TpT), but is inactive on the 3'-isomer. None of these enzymes sever the intradimer phosphodiester bond. In contrast, nuclease P1, besides removing the 5'-nucleotide from d-TpT<p>T, also attacks the intradimer phosphodiester bond of the 5'-isomer (but not of the 3'-isomer) to generate the novel compound, dT<>d-pTpT (Liuzzi *et al.*, 1989).

Digestion of UV-irradiated homopolymer poly(dA)·poly(dT) demonstrated that enzymatic treatment can be used to quantitate cyclobutane dimers in long polymers. For example, digestion of poly(dA)·poly(dT) with snake venom phosphodiesterase in the presence of calf alkaline phosphatase generates mononucleosides plus trithymidylates containing a cyclobutyl dimer at the 3'-terminus. Quantitation of these compounds gives the number of cyclobutane dimers present in UV-irradiated poly(dA)·poly(dT), for any given UV fluence, identical to that calculated from formic acid hydrolysis (Liuzzi *et al.*, 1989). However, the latter procedure, by cleaving the N-glycosyl bonds, liberates free bases and acid-stable photoproducts (Love and Friedberg, 1983), and thus such analysis gives no information as to the state of intradimer phosphodiester bonds. Alternatively, since snake venom phosphodiesterase hydrolysis leaves the phosphodiester bonds of a 3'-cyclobutane dimer-containing trithymidylate untouched, this allows detection of any such modifications.

Our later work extended this study to include trithymidylates containing a (6-4) photoproduct (Liuzzi *et al.*, submitted for publication). This was important because (6-4) photoproducts, as mentioned above, are not stable under the acidic conditions used to quantitate cyclobutane dimers. Contrary to the report that (6-4) photoproducts are stable in trifluoroacetic acid (Franklin *et al.*, 1982), this appears to not be the case as the yield achieved by this method is significantly lower than obtained by the new technique outlined in this chapter. Recently, other techniques used to measure the induction of (6-4) photoproducts relative to cyclobutane dimer (radioimmunoassay, analysis of alkali-labile sites at photoisomerized lesions, laser cytometry, and a photolyase/UvrABC endonuclease assay) have also found a much higher proportion of (6-4) photoproducts than initially reported (Mitchell, 1988b; Mitchell *et al.*, 1990a; Mori *et al.*, 1990; Thomas *et al.*, 1989).

Using the results of the trithymidylate studies, analyses were extended and an enzymatic digestion/ HPLC assay system developed which is capable of accurately quantitating lesions found in genomic DNA or excision fragments from UV-treated, [^{3}H]-thymidine-labelled cells. This procedure involves initial digestion with snake venom phosphodiesterase and calf spleen phosphatase, followed by brief treatment with nuclease P1. The end result is a series of labelled dinucleotides plus labelled thymidine, which can be readily separated by HPLC chromatography (see Figure 1). The advantages of this protocol are that both cyclobutane dimers and (6-4) photoproducts can be quantitated in the same assay (this chapter), TA* photoproducts can also be detected (see Chapter 6), and dimerized nucleosides with an intradimer phosphodiester bond can be distinguished from those in which this bond is still intact.

To test the utility of this method, experiments were first carried out on a normal human fibroblast cell strain, looking at either the disappearance of UV photoproducts from genomic DNA or the accumulation of these lesions in excision fragments, at various times after UV irradiation. All lesions accumulating in excision fragments can be accounted for by their accompanying disappearance from the genomic DNA. This, along with the reproducibility of the procedure, attests to its usefulness.

EXPERIMENTAL PROCEDURES

For more detailed experimental procedures, see Appendix A, page 254.

Materials. All tissue culture supplies were obtained from GIBCO/BRL Inc. (Burlington, ON), unless otherwise noted. Nuclease P1, DNase I, staphylococcal nuclease, and calf alkaline phosphatase were purchased from Boehringer Mannheim Canada (Dorval, PQ); snake venom phosphodiesterase was obtained from Sigma Chemical Company (St. Louis, MO); polynucleotide kinase was obtained from Pharmacia (Uppsala, Sweden); all nucleic acids were procured from Sigma; all chemicals were obtained from BDH Chemicals (Toronto, ON); and dialysis tubing (Spectra/Por) and all other general laboratory supplies were purchased from Fischer Scientific (Pittsburg, PA).

Cell strain and culture conditions. Experiments were conducted on the normal diploid fibroblast strain (GM38) established from a skin biopsy of a 9-year-old black female human subject. This strain was purchased from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ).

Cells were grown as monolayer cultures in Ham's F12 medium supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin G, and 100µg/ml streptomycin sulfate. Cultures were incubated at 37°C in a humidified (75-85%) atmosphere of 5% CO_2-95% air. Cultures were found to test negative for *mycoplasma* contamination, using the method of Schneider *et al.* (1974).

Radioactive labelling of cellular DNA. Approximately 2.5×10^6 cells were seeded in plastic Petri dishes (diameter 15 cm; Lux Scientific Corp., Newbury, CA) and incubated in Thy-free Ham's F12 (supplemented as indicated above) overnight to establish exponential growth. The cultures were incubated for an additional 48 hr in the same medium containing a final concentration of 5 µCi/ml [^{3}H]dT (1 mCi/ml; 41 Ci/mmol; NEN Canada, Montreal, PQ). The radioactive medium was then removed, and each monolayer culture subsequently washed with 10 ml of PBS. Fresh nonradioactive medium was added and cultures were incubated for 24 hr to deplete endogenous precursor pools of tritium label. In each experiment, 6 plates were assayed per time point, five for examining excision fragments and one for monitoring genomic DNA.

UV irradiation and subsequent incubation of cell cultures. After removal of the post-labelling medium, cultures were washed once with PBS and drained thoroughly. The fibroblasts were immediately exposed, in open dishes, to a UV fluence of 40 J/m^2 delivered by two 15-W germicidal (low-pressure mercury vapor) lamps (Model GE 15T8; General Electric, Toronto, ON), emitting 97% of their radiant energy at 254-nm wavelength. The incident fluence rate of the UV source was 2.3 W/m^2, as measured with a UVS Radiometer (UVP Inc., San Gabriel, CA). Six dishes of cells were sham-exposed, thereby serving as unirradiated controls. All cultures were then replenished with fresh growth medium. Six dishes of UV-irradiated cells were incubated for each of the following times: 0, 3, 6, 12, and 24 hr. Sham-irradiated dishes were incubated for 24 hr.

DNA extraction and preliminary digestion. Following UV-irradiation and incubation, GM38 fibroblasts were harvested by trypsinization and dimer-containing high-molecular-weight DNA was isolated by the method of Maniatis *et al.* (1982). Purified UV-damaged DNA had a specific activity of ~330,000 dpm/µg. Twenty µg of DNA were incubated overnight in each of 5 1.5-ml polyurethane tubes with 150 units of staphylococcal nuclease, 200 units of DNase I, 0.025 units of snake venom phosphodiesterase, and 20 units of calf alkaline phosphatase in a 1-ml reaction containing 20 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, and 2 mM $CaCl_2$. This preliminary digestion served to reduce the high-molecular-weight DNA to a series of lesion-containing trinucleotides plus mononucleosides. Since the lesion-containing trinucleotides are by far the minor species present, the mixture was subjected to dialysis overnight to remove the preponderance of mononucleosides.

Dialysis of bases, nucleosides and small oligonucleotides. A 1-ml volume containing a known quantity of radioactively labelled thymine, thymidine, the cyclobutane-dimer containing dithymidylate (d-T<p>T), or the cyclobutane dimer-containing trithymidylate (d-TpT<p>T), was subjected to dialysis, in 3 kDa MW cut-off dialysis tubing, against a large volume (~2 *l*) of ddH_20, with H_2O changes every 6 hr, for a total of 24 hr. Samples were taken at various times and the radioactivity remaining in the dialysis tubing was determined (see Figure 2). From this exploratory study, it became evident that this method could be used to remove selectively the vast excess of mononucleosides from a digested sample of UV-damaged high-molecular-weight DNA.

Dialysis of digested DNA and subsequent digestion. Prior to dialysis, the total radioactive content in each sample was determined. Samples were then dialysed, individually, in prepared dialysis tubing. Dialysis was continued for 24 hr, against a large volume of ddH_20, with H_20 changes every 6 hr. The total radioactivity remaining in each sample was determined and expressed as a percentage of initial radioactivity. Each was then subjected to the analysis outlined below.

Isolation of excision fragments. Following post-UV incubation, the medium was removed and cells were washed twice with PBS, and harvested by trypsinization. Cells were suspended in ice-cold growth medium (to inhibit the trypsin) and collected by centrifugation (1000 g for 3 min at 4°C). Each resulting ~100-µl pellet was washed twice with ice-cold PBS and recentrifuged as above. Cells were then resuspended to a total volume of 0.5 ml in 0.1 M Tris-HCl (pH 7.5) and transferred to polyurethane tubes. Two-10 µl samples of this cell suspension were added to 0.1 ml H_2O, and the cells were lysed by the addition of 0.1 ml 10% TCA, and mixed with 5 ml of EP scintillation cocktail (Beckman Instruments, Toronto, ON). The radioactive content of these samples were measured in a liquid scintillation system, and the values were used to calculate the total radioactivity of the cell sample. The remaining cell suspension was added to 0.5 ml of 10% TCA, mixed thoroughly, and the resultant cell lysate held on ice for 15 min. The TCA-soluble and -insoluble fractions were separated by centrifugation (10,000*g* for 15 min at 4°C). The acid-soluble material (~900 ul) was collected and transferred to a new polyurethane tube. The total radioactivity in the acid-soluble fraction was calculated from the radioactive content of two 20-µl sample. The remaining TCA-soluble material was extracted 5 times with 5 volumes of ice-cold diethyl ether to remove the TCA, and then taken to dryness and resuspended in 0.3 ml distilled H_2O. All samples were stored at -20°C until analyzed.

Analysis of lesion-containing trinucleotides and oligonucleotides. Each sample was subjected to 3 different treatments, and the resultant products were assayed using reverse-phase HPLC. The three treatments are described in detail below.

(1) Formic acid hydrolysis. Samples of dimer-containing oligonucleotides (0.5-1.0 x 10^4 dpm) were hydrolysed as described elsewhere (Weinfeld *et al.*, 1986). Acid hydrolysis converts the UV-irradiated DNA to free bases and acid-stable cyclobutane pyrimidine dimers. Cytosine is deaminated to uracil by this procedure; hence cytosine-containing cyclobutane dimers appear as uracil-containing dimers on the HPLC chromatograms. The (6-4) photoproducts are not stable under these conditions, and cannot therefore be quantitated using this procedure.

(2) Enzymatic hydrolysis. Samples of dimer-containing oligonucleotides ($1\text{-}2 \times 10^4$ dpm) were incubated overnight at 37°C with 0.025 units of snake venom phosphodiesterase and 25 units of calf alkaline phosphatase in a 1-ml reaction mix containing 50 mM Tris-HCl (pH 6.0), 1 mM $MgCl_2$, 1 mM $ZnCl_2$, and 5 mM β-mercaptoethanol. Six units of nuclease P1 was then added and the reaction mix incubated for an additional 1 hr at 37°C. In addition to free nucleosides, this protocol generates dinucleoside monophosphates containing a cyclobutane dimer or a (6-4) photoproduct (see below). Half of the enzymatically hydrolysed sample was applied directly to the reverse phase column. The remaining 0.5 ml was subjected to alkaline hydrolysis (see below).

(3) Alkali treatment. Enzymatically hydrolysed samples (~0.5 ml) were supplemented with 55 µl 2 N NaOH (final concentration ~0.2 N), and incubated at 90°C for 90 min in a 1.5-ml polyurethane tube. Upon cooling, the sample was then neutralized with 55 µl of 2 N HCl and examined by HPLC. This procedure hydrolyzes (6-4) photoproducts, thereby altering their retention time on reverse-phase HPLC.

(4) HPLC analysis. The instrumentation used for the reverse phase chromatographic analysis has been described elsewhere (Liuzzi *et al.*, 1989). The separation of radioactive species was performed on a Whatman Partisil-10 ODS-2 column (250 x 4.6 mm i.d.) using the following gradient elution conditions: 100% buffer A [50 mM NaH_2PO_4 (pH 4.5)] and 0% buffer B [50 mM NaH_2PO_4 (pH 4.5), containing 50% methanol] for 1 min followed by a linear gradient from 100% to 0% buffer A and from 0% to 100% buffer B over 30 min. The 100% buffer B was maintained for 5 min followed by a return to initial conditions in 4 min. The flow rate was 1 ml/min. Seventy 0.5-ml fractions were collected; 5 ml of scintillation cocktail was added to each fraction and its radioactivity content was determine. The identity of several of the radioactive species under study (Thy, dT, T<>T, T<>U, d-T<p>T, and d-T<p>C) was determined based on their retention time as ascertained by others in our laboratory using the identical system (Weinfeld *et al.*, 1986; Liuzzi *et al.*, 1989). The authenticity of the dimerized nucleosides, with a cleaved intradimer phosphodiester bond (dT<>dT and dT<>dC), and of the (6-4) photoproduct-containing dinucleotides (d-T[p]T and d-T[p]C) were determined as described below.

Characterization of d-T[p]T, d-T[p]C, dT<>dT, and dT<>dC. The authenticity of compounds containing (6-4) photoproducts was ascertained by hot alkali treatment. Material was collected from the HPLC as indicated above. Again, one-third of the sample was reinjected onto the HPLC and a second aliquot was subjected to formic acid hydrolysis. The remaining portion of the sample was treated with hot alkali as indicated above for excision fragments. Formic acid hydrolysis confirmed that the compound in question did not contain cyclobutane dimers. However, hot alkali treatment hydrolyses (i) the *N*-glycosyl bond of the 3'-pyrimidine of the (6-4) photoproduct, and (ii) the phosphodiester bond between the cross-linked pyrimidines of the (6-4) photoproduct (Franklin and Haseltine, 1982). This results in a less hydrophobic compound and as expected, moved the material in question to the beginning of the HPLC chromatogram (see Figure 3).

Radioactive peaks corresponding to modified dimer-containing dinucleosides (dT<>dT and dT<>dC) were identified by photochemical reversal. Suspect material was collected from the HPLC, taken to dryness in a SpeedVac concentrator (model SVC 100H; Savant Instruments, Inc., Farmingdale, NY), resuspended in 1 ml of H_20, and divided into three aliquots. One aliquot was reinjected onto the HPLC. A second was subjected to formic acid hydrolysis. The remainder was exposed to a photoreversing fluence (5500 J/m^2) of 254-nm light (Haseltine *et al.*, 1980) prior to reinjection onto the HPLC (see Figure 4 for dT<>dT). Formic acid hydrolysis established that the material contained cyclobutane dimers. The later procedure caused a shift in the retention time of the radioactivity to that of thymidine. (The DNA contained a label on thymidine only and thus cytidine could not be detected.) Since monomerization of the cyclobutane dimer produced a mononucleoside, the compound in question must contain a modified dimer, as photochemical reversal of an intact dimer would produce d-TpT or d-TpC.

Analysis of the solubility of the small oligonucleotide, $p(dT)_{10}$, in 5% TCA. $(dT)_{10}$ was dissolved in water at a concentration of 1 nmol/ml. Fifty pmol of DNA was incubated for 1 hr at 37°C in a 50-ul reaction containing 10 mM Tris-acetate (pH 7.0), 10 mM magnesium acetate, 50 mM potassium acetate, 5 µCi of $[\gamma\text{-}^{32}P]$ATP, and 20 units of T4 polynucleotide kinase. An aliquot (~5000 cpm) of the labelled oligonucleotide was then diluted with ddH_20 (or a solution containing 2 mg/ml BSA or 5×10^6 human fibroblasts) to a final volume of 0.5 ml, and supplemented with an equal volume of 10% TCA. The acid-soluble fraction was collected and its radioactive content determined as above.

Strong anion exchange (SAX) chromatography. Each enzymatically digested or TCA-soluble sample derived from post-UV incubated cells was diluted to 1 ml with distilled H_2O in order to lower the salt concentration of the sample to an acceptable level, since salt interferes with the retention of molecules on an SAX column. Analysis was conducted on a Whatman Partisil 10 SAX column (250 x 4.5 mm i.d., Whatman Inc., Clifton, NJ). Elution conditions were as follows: 100% buffer A [0.1 mM KH_2PO_4 (pH 6.6) containing 10% ethanol] and 0% buffer B [0.4 M KH_2PO_4 (pH 6.6) containing 10% ethanol] for 5 min followed by a linear gradient from 100 % to 0 % buffer A and from 0 % to 100 % buffer B over 30 min. The 100 % buffer B was maintained for 10 min followed by a return to initial conditions in 10 min. The flow rate was 0.8 ml/min. Eighty 0.5-ml fractions were collected and their radioactivity content determined as described above. The retention time of various radioactive species under study [Thy, dT, dT<>dT, d-TpT, dTpTpT, $(dT)_5$, $(dT)_7$, and $(dT)_{10}$] were determined with authentic markers or after verification of structure (dT<>dT) as described above.

Polyacrylamide gel electrophoresis. A denaturing polyacrylamide gel (20% acrylamide, 45% w/v urea) was made as outlined in Maniatis *et al.* (1982). Excision fragment-containing samples were subjected to [^{32}P] end-labelling, utilizing T4 polynucleotide kinase in the presence of [γ-^{32}P] ATP, and subsequently electrophoresed on the vertical gel at 800 V for 4 hr. The glass plates were then separated, and the gel was transferred to filter paper and encased in plastic wrap. The bands were visualized after overnight exposure to X-ray film. The length of the oligonucleotides in question was determined by a series of [^{32}P] end-labelled markers which had been run in parallel lanes.

RESULTS

Use of dialysis to remove mononucleosides from digested DNA. Although the advertised molecular weight cut-off (<3000 Da) of the dialysis tubing employed is greater than the lesion-containing trinucleotides (MW <1000 Da), these molecules could be preferentially retained under the conditions used (see Figure 2). Thymine and thymidine were lost rapidly (approximately only one-third remains after 1 hr of dialysis) whereas d-T<p>T passed through the membrane at a slower rate (~50% loss after ~10 hr). The retention of the trinucleotides appeared to be based on the charge of the species in question. Essentially 100% of d-TpT<p>T was retained. Therefore, this was an effective procedure for reducing the large pool of mononucleosides from a enzymatically digested sample of UV-treated DNA. This procedure proved to be invaluable, not only for analyzing lesions present in genomic DNA, but also for analyzing excision fragments isolated from *E. coli*. These cells had very large pools of free thymidine which otherwise restrict the examination of these small lesion-containing oligonucleotides (see Chapter 7).

Table I summarizes the percentage of radioactivity that was retained when [^{3}H] thymidine-labelled post-UV incubated genomic DNA was first digested with snake venom phosphodiesterase and calf alkaline phosphatase, and subsequently dialysed overnight against a large volume of water. These values were used as part of the calculation scheme employed to determine the number of lesions present in the genomic DNA. Only about 0.5% of the total radiolabel was retained under these conditions. The retained material presumably represented concentrated trinucleotides, although subsequent hydrolysis of this material, as well as the 0.36% of total radiolabel retained in the case of sham-irradiated cells, revealed that there was [^{3}H] thymidine present which was not associated with the trinucleotides. That all of the trinucleotides had remained in the dialysis tubing was demonstrated by the excellent agreement of the number of lesions induced in the genomic DNA as calculated using these data (see below) with what has been previously published (Paterson *et al.*, 1981; Mitchell *et al.*, 1985).

Formic acid hydrolysis of UV-treated genomic DNA or excision fragments collected from post-UV incubated normal human fibroblasts. The standard assay for quantitating cyclobutane dimers in DNA is by formic acid hydrolysis. However, this method has the major drawback that it does not allow enumeration of (6-4) photoproducts as these lesions are labile under such conditions. It also does not allow discrimination of the status of the intradimer phosphodiester bond as this procedure ruptures the *N*-glycosyl bond to yield two bases joined by a cyclobutane bridge. However, since this is a tried and true method for quantitating cyclobutane dimers, this procedure was carried out on both genomic DNA and excision fragments to serve as a frame of reference for the new enzymatic hydrolysis protocol. For example, as seen in Figure 5A, this methodology showed that the sample in question [trinucleotides prepared from unrepaired UV-treated (40 J/m^2) genomic DNA] contained 8.0% T<>U dimers (cytosine is deaminated to uracil) and 21.5% T<>T dimers. As seen below, this correlated well with values obtained from enzymatic hydrolysis.

Separation of SVP/CAP/NP1 digested unrepaired UV lesion-containing DNA into distinct species by HPLC chromatography and identification of (6-4) photoproducts. After isolating the lesion-containing trinucleotides by preliminary digestion and dialysis of genomic DNA, nuclease P1 was added to reduce the trinucleotides to dinucleotides plus mononucleotides. These species were separated on reverse-phase HPLC giving radioactive profiles like that shown in Figure 5B. The retention times of the two labelled cyclobutane dimer-containing dinucleotides, d-T<p>C and d-T<p>T, were verified with authentic markers and represented 7.9% and 21.8% of total radioactivity, respectively, for cells which were not allowed to undergo repair. Within the limits of experimental error, formic acid and enzymatic hydrolysis gave equivalent results for the measurement of cyclobutane dimers (compare Figure 5A and 5B), and compared favorably with previously published results (Paterson *et al.*, 1981).

A novel peak, which eluted at fraction 30, was conceived to be (6-4) photoproducts. To test this supposition, the fractions containing this material were isolated and purified as indicated in Experimental Procedures. The pooled fractions were split into three aliquots, one of which was reinjected onto the HPLC to insure that the purification procedure had not altered the compound (Figure 3A). The second aliquot was subjected to formic acid hydrolysis, the results of which are seen in Figure 3B. The majority of radioactivity was in thymine (59.6%), with small peaks eluting at around fractions 46 and 52. These latter peaks likely contained (6-4) photoproduct-dimerized bases. Although these lesions are not stable enough in hot acid to allow their quantitation, formic acid hydrolysis of every sample which contained a high proportion of (6-4) photoproducts generated these peaks. They were, however, not further characterized.

(6-4) photoproducts are sensitive to hot alkali. This treatment produces a less hydrophobic compound due to hydrolysis of (i) the *N*-glycosyl bond of the 3'-pyrimidine of the

(6-4) photoproduct, and (ii) the phosphodiester bond between the cross-linked pyrimidines of the (6-4) photoproduct (Franklin *et al.*, 1982). The remaining aliquot of the supposed (6-4) photoproduct peak was therefore subjected to this treatment, and caused the radiolabel to move to fractions 7 - 12 on the chromatogram. This shift was predicted if this material in fact contained (6-4) photoproducts. Further support as to the identity of the (6-4) photoproduct peak later came from Liuzzi *et al.* (submitted for publication) by demonstrating that this peak was fluorescent at certain wavelengths (Lippke *et al.*, 1981) and prone to photolysis (Taylor *et al.*, 1988).

Hot alkaline treatment was, in general, used to confirm the identity of (6-4) photoproducts in enzymatically hydrolysed samples, as illustrated in Figure 5C. In this instance, a portion of the digestion mixture used to generate the chromatogram shown in Figure 5B was treated with hot alkali prior to HPLC analysis. This resulted in a shift of the (6-4) photoproduct peak from fraction 30 (in Figure 5B) to the beginning of the chromatogram (in Figure 5C), without affect on the mobility of the cyclobutane dimer-containing peaks.

Separation of SVP/CAP/NP1 digested excision fragments into distinct species by HPLC chromatography and identification of modified cyclobutane dimers. Having established the utility of enzymatic hydrolysis as outlined above, this strategy was then employed to determine photoproduct frequency in excision fragments isolated from post-UV (40 J/m^2) incubated (24 hr) cells. Excised lesion-containing DNA fragments are retained inside a mammalian cell for at least 24 hr and can be collected in the TCA-soluble fraction of post-UV incubated cell cultures (La Belle and Linn, 1982; Weinfeld *et al.*, 1986). As expected, the percentage of [^{3}H]-thymine label in this fraction from normal human fibroblasts increased steadily with post-UV incubation (see Table IV), reflecting active excision of UV damage. As a first step, these excision fragments were subjected to formic acid hydrolysis as seen in Figure 6A. This revealed that the 24-hr-repair sample contained 13.6% and 23.0% of the total radiolabel recovered as T<>U and T<>T cyclobutane dimers, respectively, which was in good agreement with previous reports (La Belle and Linn, 1982; Weinfeld *et al.*, 1986). Enzymatic hydrolysis and subsequent HPLC of an aliquot of the same sample resulted in a radioactive profile characterized by only 4.5% of counts in d-T<p>C and 4.1% in d-T<p>T (Figure 6B). There was also a peak representing (6-4) photoproducts that contained 19.3% of total radiolabel. However, hot alkali treatment demonstrated that there was some other (novel) species co-migrating with the (6-4) photoproducts

(Figure 6C) as 9.2% of the total radioactivity remained in the same position after this treatment. Comparative examination of the resulting chromatogram (Figure 6C) with its nonalkaline-treated counterpart (Figure 6B) clearly indicated that ~10% of the acid-soluble radioactivity was in (6-4) photoproducts, and ~9% represented a different species. Hot alkali treatment was not employed, however, for quantitating (6-4) photoproducts since other minor unidentified compounds were found to elute at the retention time of the alkali-hydrolysed (6-4) photoproducts (data not shown). Although the (6-4) photoproducts co-migrated with another species, namely dT<>dC (see below), the contribution of the former lesions was nonetheless easily calculated as the percentage of counts in dT<>dC, d-T[p]Py, and d-T<p>C, minus the percentage of counts in T<>U given by the formic acid analysis.

HPLC analysis of the enzymatically hydrolysed 24-hr-repair excision fragments also revealed another novel species (representing 17.1% of total counts) eluting at fraction 36. Due to the low number of intact cyclobutane dimers in the sample as compared to the value found by formic acid hydrolysis, and because of previous work (Weinfeld *et al.*, 1986) suggesting the presence of cyclobutane dimers with a cleaved interphosphodiester bond in excision fragments, the hypothesis that the two novel peaks represented the labelled modified dimer-containing dinucleotides, dT<>dC and dT<>dT, was entertained. To test this supposition, suspect material was purified by HPLC and analyzed as indicated in Experimental Procedures. Results from a series of experiments utilizing presumably dT<>dT, which elutes at fraction 36, are shown in Figure 4. Reinjection of the same material demonstrated that the isolation procedure had not altered the retention time of the species under study (Figure 4A). Formic acid hydrolysis of such a sample confirmed that the material in fact contained a cyclobutane dimer, in particular, T<>T (Figure 4B). As the two pyrimidines in a modified dimer are connected only by the cyclobutane bridge, breakage of the bridge would result in the release of two mononucleosides. Thus, an aliquot of this sample was exposed to 5500 J/m^2 of 254-nm light. Under these photochemical reversing conditions, ~70% of the radioactivity was converted to thymidine (Figure 4C), supporting the expectation that this material constituted modified thymidine-containing cyclobutane dimers. A similar study confirmed that the species co-migrating with the (6-4) photoproducts was the other labelled modified dimer, dT<>dC (data not shown).

Having established the molecular configuration of the compounds in the various peaks on the HPLC profile, the number of cyclobutane dimers in the sample as measured by formic acid hydrolysis did, in fact, agree with that obtained by enzymatic hydrolysis of excision fragments.

Moreover, the latter procedure had the distinct advantage that the state of the intraphosphodiester bond of a cyclobutane dimer could be ascertained. Although there were modified dimers in a sample of excision fragments, these did not appear in a sample of UV-irradiated genomic DNA (no repair). This leads to the important conclusion that it was not the enzymatic digestion protocol that was producing modification of these lesions.

Removal of photoproducts from genomic DNA of post-UV incubated normal human fibroblasts. Having established the utility of the new protocol for quantitating cyclobutane dimers and (6-4) photoproducts in small DNA fragments, this procedure was used to analyze genomic DNA isolated from normal human fibroblasts after various post-UV incubation times. It should be kept in mind that the UV fluence to which cells were subjected in this study (i.e. 40 J/m^2) represents a dose at which less than 0.1% of the cells can form colonies (Smith and Paterson, 1982). Furthermore, although excision repair continues in these non-dividing cells, 40 J/m^2 is well above the repair saturating fluence of ~15 J/m^2 (the D_{10} value for GM38 fibroblasts) for at least cyclobutane dimer removal (Smith and Paterson, 1982). Results presented below indicate that (6-4) photoproduct repair is not saturated below 40 J/m^2.

[^{3}H] thymine-labelled dimer-containing trinucleotides were prepared and subjected to the three basic treatments outlined above, namely, formic acid hydrolysis and enzymatic hydrolysis either alone or followed by hot alkaline treatment. The percentages of radiolabel representing the various UV photoproducts for repair times of 0, 3, 6, 12, and 24 hr, as determined by this procedure, are summarized in Table II, and depict the average of three experiments. (Values from any one experiment were within 15% of the average for peaks representing less than 2% of the total radioactivity in the sample, and within 5% for photoproduct peaks representing greater than 12%.) Table II also includes values for sham-irradiated cultures. These revealed any peaks with retention times identical to those of the UV photoproducts, and thus, if observed, were considered to be background noise. It was clear from this work that there was only a small amount of unidentified background in the locations of (6-4) photoproducts and d-T<p>T (0.6% and 0.1% of the total radioactivity, respectively). These almost certainly represented radiolysis compounds.

The percentage of radiolabel representing the various UV photoproducts generally decreased with post-UV incubation time. This was most evident for (6-4) photoproducts, which decreased from 3.3% to 0.5% of total radioactivity over the maximal (24-hr) period examined. Although these data showed the general trend of repair, it was much more meaningful to identify the absolute number of lesions induced by the irradiation conditions, and those remaining after various repair times. These have been calculated and are summarized in Table III. Values were computed by multiplying the percentage of radioactivity which remained in the dialysis membrane with (i) the percentage of label in each peak (Table II) and (ii) the number of thymidine residues present in the human genome (taken as 3.19 x 10^9). In calculating the number of T^T dimers, this figure was divided by 2 as either thymidine could be labelled. The number of thymidines/genome was calculated based on the assumptions outlines in the legend which accompanies Table III.

The results indicated that a fluence of 40 J/m^2 induced ~ 1.64 x 10^6 and 1.21 x 10^6 T^T and T^C dimers per human genome, respectively. Based on the assumption that C^C dimers occur with a frequency of 0.24 x T^C frequency (Weinfeld *et al.*, 1986), the total number of cyclobutane dimers induced is ~3.1 x 10^6, a value in keeping with previously published determinations (Paterson *et al.*, 1981). By 24 hr, there still remained 1.44 x 10^6 T^T dimers and 0.68 x 10^6 T^C dimers per cell.

After subtracting background, the number of T[p]C and T[p]T (6-4) photoproducts induced by this UV fluence was calculated to be 4.3 x 10^5 per genome. Extrapolation of data accumulated on the frequency of the various UV lesions occurring at specific sequences in irradiated *E. coli* (Brash and Haseltine, 1982) and human cells (Brash *et al.*, 1987) indicated that C[p]C (6-4) photoproducts occur with a frequency of 0.27 x (T[p]C + T[p]T). {C[p]T photoproducts do not form (Brash and Haseltine, 1982; Brash *et al.*, 1987).} Therefore, the total number of (6-4) photoproducts present immediately upon irradiation with 40 J/m^2 was 5.5 x 10^5 per cell, representing ~18% of the frequency of cyclobutane dimers. By 6 hr there remained only 8.9 x 10^4 (6-4) photoproducts per genome and by 12 hr they had essentially been removed entirely.

Accumulation of photoproducts in excision fragments from post-UV incubated normal human fibroblasts. Data such as that illustrated in Figure 6, as well as that from other repair times, are summarized in Table IV. Again, these results represent the average of three experiments. As the percentages presented in Table IV directly reflected the absolute number of UV lesions excised from the genomic DNA, it was evident that, whereas cyclobutane dimers continued to accumulate in excision fragments at least up to 24 hr, (6-4) photoproducts plateaued by 6 hr. In addition, it was apparent that the majority of cyclobutane dimers were present in these small oligonucleotides as modified dimers even at 3 hr post UV, although the proportion was lower at the earlier repair time (see below).

As for the disappearance of lesions from genomic DNA, the values outlined in Table IV were used to calculate the absolute yield of cyclobutane dimers and (6-4) photoproducts in excision fragments isolated from post-UV incubated cells, and are summarized in Table V. The assumptions used for these calculations are indicated in the legend of Table V. From these data, it can be estimated that each normal GM38 cell excised ~4.4 x 10^5 T^T and 5.2 x 10^5 T^C cyclobutane dimers during a 24 hr post-irradiation incubation, a result consistent with our previous work (Weinfeld *et al.*, 1986). If, in fact, C^C dimers occur with a frequency of 0.24 x T^C frequency, the total number of cyclobutane dimers excised by 24 hr after 40 J/m² was ~1.1 x 10^6. During the same repair period, a normal cell excised ~4.2 x 10^5 T[p]C and T[p]T (6-4) photoproducts. Given a C[p]C frequency of 0.27 x (T[p]C + T[p]T) (based on Brash and Haseltine, 1982 and Brash *et al.*, 1987), this corresponds to excision of ~5.3 x 10^5 (6-4) photoproducts in total. Unlike cyclobutane dimers, the level of these latter lesions excised per cell at 6 hr was equal to that at 24 hr, suggesting that (6-4) photoproduct repair was complete within 6 hr. This finding confirms and extends recent data by others (Mitchell *et al.*, 1985; Mori *et al.*, 1990). These investigators have found that repair is complete by 6 hr following 4 or 10 J/m² for this class of UV lesions. This implies that, unlike cyclobutane dimer removal which is saturated by 15 J/m² (Smith and Paterson, 1982), (6-4) photoproduct repair does not reach saturation at less than 40 J/m².

There was a general trend for the number of modified cyclobutane dimers (i.e., those lacking an intradimer phosphodiester linkage) found in excision fragments to increase with post-UV incubation time more rapidly than did unmodified dimers. Although, after 3 hr repair, modified dimers represented ~60% [data taken from Table IV: (3.0+1.4)\ (3.0+1.4+1.2+1.7)] of the total cyclobutane dimers which had been excised, it was notable that the percentage of total counts in unmodified dimers scarcely increased after 6 hr post UV (see Table IV). However, there was a dramatic increase in the representation by modified cyclobutane dimers. Thus, from 6 to 12 to 24 hr post UV, the percentage of cyclobutane dimers that were modified increased from 48% to 58% to 77%.

Kinetics of UV photoproduct repair in normal human fibroblasts. The results in Tables III and V have been reiterated in Figures 7 (for cyclobutane dimer repair) and 8 [for (6-4) photoproduct removal]. The total number of both classes of lesions induced into the DNA by 40 J/m², and the subsequent repair, is in keeping with published data from our and other laboratories (Paterson *et al.*, 1973; Mitchell *et al.*, 1985; Mitchell and Nairn, 1987), although the initial (6-4) photoproduct estimates are significantly lower than those measured by others (Thomas et al., 1989; Mitchell et al., 1990c). This discrepancy is discussed in detail below. Critical to the validation of the present methodology, the lesions removed from genomic DNA could be accounted for, within experimental error, by their appearance in excision fragments.

State of the interpyrimidine phosphodiester bond in a (6-4) photoproduct. Since cyclobutane dimers found in excision fragments represented two discrete molecular species, it was of interest to determine whether or not the same was true for the (6-4) photoproducts. To answer this question, the TCA-soluble material from post-UV incubated human cells was subjected to (i) enzymatic hydrolysis or (ii) formic acid hydrolysis, and subsequent analysis by reverse-phase or SAX HPLC. The formic acid procedure provided the total number of cyclobutane dimers present in each sample (Figure 9A). The various photolesion-containing dinucleotides(sides) released by enzymatic hydrolysis were retained differently on the two types of columns employed (Figure 9B). The labelled thymidine-containing molecules were resolved into five peaks by reverse-phase chromatography, as discussed previously. On a SAX column, charged species are retained to varying degrees depending on the total charge of the molecule. Uncharged species have no affinity for the column matrix and therefore elute in the void volume. As illustrated in Figure 9C, the percentage of radiolabel not retained by the SAX column after enzymatic hydrolysis was 68.7%. Since the expected percentage of counts in uncharged species, if the excised (6-4) photoproducts were charged, was the same as the observed value (see calculations in the legend to Figure 9), it could be concluded that the (6-4) photoproducts were retained on the SAX column and therefore had at least one charge. Since the reaction mixture included alkaline phosphatase, and since these molecules had been reduced to dinucleotides, this phosphate group must be located internally and form part of an intact phosphodiester linkage.

Length of excision fragments found in post-UV incubated normal human fibroblasts. Because of the uncertainty in the ability to recover all the excised photoproducts from post-UV incubated cells, a series of experiments was performed on a [^{3}H] thymidine-labelled oligonucleotide [$p(dT)_{10}$] to determine its solubility in 5% TCA under various conditions (see Table VI). Prior irradiation appeared to increase solubility (52% as compared to 28% for irradiated versus untreated oligomer). Furthermore, the presence of protein (BSA) or cells (at approximately the same concentration as would be used in the experiments outlined in this chapter) increased the solubility tremendously (86 and 72%, respectively). However, as there was not complete recovery of the 10-mer under these conditions, this implies that the excision fragments must have been smaller than 10 nucleotides in length, because the repair kinetics for genomic DNA and excision fragments indicated that the excised UV photoproducts were being recovered in full. This is in concordance with previous work from our laboratory (Weinfeld *et al.*, 1986).

To investigate the length of excision fragments, TCA-soluble material from various repair times was analyzed by SAX HPLC chromatography. This column type retains molecules based solely on their charge and is independent of base composition. Figure 10 illustrates the SAX profile for the TCA-soluble fraction from 12 hr post-UV incubated cells. Markers of known length are indicated on the diagram. The majority of radioactivity (~55%) peak was in the void volume, where uncharged molecules such as thymine and thymidine elute. Further analysis of this material disclosed that although the bulk was thymidine, there were also some dT<>dT and dC<>dT present, although it was only a small fraction (8%) of the total number of modified dimers found in the TCA-soluble material (data not shown). There was also a distinct peak (~34% of total radiolabel) which co-migrated with the $(dT)_7$ marker. (This 7-mer had been dephosphorylated and therefore contained 6 internal charges.) A small peak (~9% of total counts) co-migrated with the $(dT)_5$ marker. These results were confirmed by polyacrylamide gel electrophoresis (PAGE). After [^{32}P] end-labelling the TCA-soluble fraction from post-UV incubated cells, this material was electrophoresed on a 20% polyacrylamide gel. As seen in Figure 11 for 12-hr repair, the major species present corresponded to 7 nucleotides in length, with some 6-mers and 5-mers present. This length of excision fragments, and the proportions in the various peaks (as seen on SAX or PAGE) were, within experimental error, independent of post UV repair time (data not shown).

DISCUSSION

The techniques employed in the past to measure the repair of UV photoproducts all have shortcomings, as presented in the introduction of this chapter. Therefore, in order to characterize and quantitate more precisely UV-induced lesions present in both genomic DNA and excised oligonucleotides, a new methodology was necessary. The assay system introduced here involves enzymatic hydrolysis of either the genomic DNA or excision fragments collected from post-UV incubated cells, and is not completely unprecedented (Patrick and Rahn, 1976). Trinucleotides produced upon snake venom phosphodiester digestion of UV-irradiated DNA have been previously studied, but such analysis was complicated by the 5'-nucleotide and was therefore of limited value (Setlow *et al.*, 1964; Delleweg and Wacker, 1964). The current technique, by hydrolysing the 5'-nucleotide with nuclease P1, solves this problem as well as many of the inadequacies of other repair measurement techniques. For example, although formic acid hydrolysis enables accurate quantitation of cyclobutane dimers (Love and Friedberg, 1983), this technique does not allow measurement of (6-4) photoproducts as the latter are unstable under these conditions (Wang and Varghese, 1967). Similarly, UV endonucleases are specific for cyclobutane dimers (Gordon and Haseltine, 1980), and measuring the disappearance of such sites in post-UV incubated genomic DNA gives no information regarding (6-4) photoproducts. In contrast, the strategy presented here provides a simple means to recover quantitatively both cyclobutane dimers and (6-4) photoproducts. Hot alkaline hydrolysis of the enzyme-digested samples subsequently serves to assess the authenticity of the (6-4) photoproducts (Figure 3), and to separate the two co-migrating species, namely (6-4) photoproducts and the thymidine-cytidine-containing modified dimer.

As ascertained by this new protocol, the total number of lesions induced into the DNA of a single human fibroblast by 40 J/m^2 of 254-nm light was 1.21 x 10^6 T^C, 1.64 x 10^6 T^T, and 0.43 x 10^6 T[p]Py. Assuming that C^C lesions are present at a frequency of 0.24 x T^C (Weinfeld *et al.*, 1986), the total number of cyclobutane dimers induced was 3.14 x 10^6/cell or 7.9 x 10^4/J/m^2/cell (assuming a linear dose response), in keeping with other published data (Paterson *et al.*, 1973; Cadet *et al.*, 1983). Given a C[p]C frequency of 0.27 x (T[p]C + T[p]T) (Brash and Haseltine, 1982; Brash *et al.*, 1987), 0.55 x 10^6 (6-4) photoproducts were induced per cell under these conditions, or 1.4 x 10^4/J/m^2/cell. This, furthermore, implies that (6-4) photoproducts occur at ~18% the frequency of cyclobutane dimers, also in approximate agreement with other studies

(Mitchell and Clarkson, 1984; Liuzzi *et al.*, submitted to for publication). However, there is currently some controversy as to the relative induction of these two major photoproducts. At very low fluences of 254-nm light (less than 2 J/m^2), Mitchell (1988a) has reported (6-4) photoproduct induction at a frequency of ~33% of cyclobutane dimers. *In vitro* irradiation of plasmid DNA, with up to 10 J/m^2, followed by immunoprecipitation with anti-(6-4) photoproduct or anti-dimer antibodies, yielded induction rates of 0.6 (6-4) photoproducts and 1.2 cyclobutane dimers/10^8 Da/J/m^2, or 50% (6-4) photoproducts relative to the more prevalent dimer (Mitchell *et al.*, 1990c). However, a value of 23% for plasmid DNA emerged from analysis of photoinduced alkali-labile sites (Mitchell *et al.*, 1990b). In yet another study, utilization of the incising activity of the UvrABC endonuclease on UV-irradiated (20-60 J/m^2) photolyase-treated CHO DNA led to the conclusion that (6-4) photoproducts occur at ~ 66% of the frequency of cyclobutane dimers in the dihydrofolate reductase gene (Thomas *et al.*, 1989). However, as outlined below, these different values likely reflect either innate variation in DNA sequence and structure or unsound assumptions of the technique employed.

It is clear that sequence influences the spectrum and frequency of UV lesions (Brash and Haseltine, 1982; Brash *et al.*, 1987) and may in part explain the considerable variation in (6-4) photoproduct induction reported for different species (Patrick and Rahn, 1976; Patrick, 1977). In addition, it has recently been reported that although cyclobutane dimer formation occurs uniformly throughout the human genome, (6-4) photoproduct induction is significantly affected by chromatin structure (Mitchell *et al.*, 1990d). By separating the nucleosomal from the internucleosomal DNA, it was determined that (6-4) photoproducts occurred 6 times more frequently in linker as compared to core DNA. Considering that both *in vitro* irradiated and prokaryotic DNA lack chromatin structure, and taking into account that approximately 24.5% of the DNA is represented by linker sequence, the remaining 75.5% being involved in nucleosome organization, a 50% frequency for (6-4) photoproducts relative to cyclobutane dimers in plasmid DNA (the most recent estimate based on an immunoassay) would translate into a relative frequency of (24.5% x 50%) + (75.5% x 50/6%) = 18.5% for eukaryotic DNA. This value correlates extremely well with numbers achieved by the methodology reported here, implying that the discrepancy between the (6-4) photoproduct induction rate reported by Mitchell *et al.* 1990c and presented here merely reflects chromatin structure.

On the other hand, the report that (6-4) photoproduct induction occurs at 66% of the frequency of cyclobutane dimers (Thomas *et al.*, 1989) is based on assumptions that are not valid.

The primary aim of the latter investigation was to illustrate preferential repair of (6-4) photoproducts in the dihydrofolate reductase (DHFR) gene of Chinese hamster ovary cells, by quantitating the total incisions generated by the UvrABC endonuclease on post-UV, photolyase-treated DNA. During this study, a comparison of T4 endonuclease sites to UvrABC-induced incisions following exhaustive photolyase treatment indicated that these latter single-stranded breaks occurred with about ~33% of the frequency of T4 endonuclease-induced breaks. The authors estimated that under the *in vitro* conditions used, only one-half of the available adducts are cleaved by the UvrABC endonuclease. Assuming that the non-dimer photoproducts were almost exclusively (6-4) photoproducts, the conclusion was made that in this region of the CHO genome, 20-60 J/m^2 of 254-nm light induces (6-4) photoproducts at 66% of cyclobutane dimers. This deduction has three major shortcomings. Firstly, the assumption that non-(6-4) photoproduct adducts contribute insignificantly to the non-dimer photoproducts is not valid. Although this inference has been drawn from a previous investigation (Myles *et al.*, 1987), careful re-analysis of these data indicates that this is not the complete story. Secondly, the assumption made by Myles *et al.* (1987), that piperdine-generated sites correspond solely to (6-4) photoproducts, is not accurate. Finally, the two studies have not used comparable irradiation conditions. An explanation of why these assumptions do not hold, and therefore why their estimated (6-4) photoproduct induction frequency cannot be accurate, is outlined below.

In the more recent work of Myles and coworkers (1987), a *Nru*I - *Hin*FI restriction fragment isolated from UV-irradiated pUNC plasmid, containing a uniquely labelled end, was treated with UvrABC in the presence of photolyase. This protocol revealed a series of bands corresponding to the incision points downstream (or upstream) of a photoproduct and was employed to illustrate aberrant cleavage by the UvrABC complex. However, in re-examining their data, this treatment also produced a discernible cleavage product corresponding to the presence of a photoproduct at the position of the $T_{116}A_{117}$ dinucleotide. This indicates that TA* photoproducts are also recognized by the UvrABC endonuclease. Although only a minor band corresponds to a lesion at this site, it is known that the frequency of induction of both (6-4) photoproducts and cyclobutane dimers in dependent on the neighboring sequence and varies substantially from one site to another (Brash and Haseltine, 1982; Brash *et al.*, 1987). Therefore, from this data alone, it cannot be concluded that TA* adducts are insignificant in number. Furthermore, all other T-A sequences in this *Nru*I - *Hin*FI fragment are juxtapositioned to a T-T or T-C sequence [where a cyclobutane dimer or (6-4) photoproduct can form], and it is therefore impossible to relate incision products in this region to a photoproduct at the T-A sequence. However, it is significant that, although all five of the dinucleotide sequences examined in detail in this study show aberrant UvrABC incisions, the position showing the greatest variation in the 5'- or 3'-cleavage site is the only one of these five dipyrimidine sequences with a T-A juxapositioned (i.e. the sequence at this site is T-T-A). This raises the distinct possibility of the presence of TA^* photoproducts at the neighboring site. In addition, quantitation of TA^* lesions in UV-irradiated (40 J/m^2) human DNA, using the enzymatic digestion protocol introduced in this chapter, indicates that these lesions are induced at 50% of the frequency of (6-4) photoproducts (see Chapter 6).

In conjunction with the UvrABC endonuclease assay, (6-4) photoproducts are typically confirmed by treatment with hot 1 M piperidine. This procedure presumably generates single-stranded breaks specifically at (6-4) photoproducts (Myles *et al.*, 1987). The use of this technique is based on the work of Lippke *et al.* (1981), who detected (6-4) photoproducts at pyrimidine nucleoside-cytidine sequences by their alkaline-lability. Careful analysis of the data presented in this latter study clearly shows that UV-irradiation of DNA also generated alkali-sensitive sites at T-A sequences. Furthermore, piperidine treatment of the UV-irradiated restriction fragment studied by Myles *et al.* (1987) produced distinct doublets at two different A-T-C sequences, implying the existence of an alkaline-labile A-T photoproduct. Piperidine cleavage products were also generated at T-A sites, although these are much less prevalent.

A further difficulty in drawing a conclusion regarding the identity of non-dimer UV photoproducts from the study of Myles and coworkers (1987) is that this investigation involves fluences of UV light significantly higher than what has been used by Thomas *et al.* (1989) (4000 J/m^2 versus 20-60 J/m^2). It is not known at this time how the rate of induction of other minor photoproducts, such as TA^* lesions, thymine glycols, photohydrates, as well as purine damage (Patrick and Rahn, 1976) varies with fluence.

Besides assigning a relative induction rate for (6-4) photoproducts which does not comply with the findings of others, an additional discrepancy between the results generated by the UvrABC endonuclease assay and accumulated data of several other studies indicates that (6-4) photoproducts are not the only significant non-dimer adduct recognized by the UvrABC complex. This protocol reveals repair kinetics for (6-4) photoproducts which are significantly slower compared to estimates obtained by two other independent techniques (Mitchell *et al.*, 1985; this study). Whereas it has been extensively reported that (6-4) photoproducts are repaired in their entirety by 6 hr post UV (10 J/m^2) in both human and rodent cells (Mitchell *et al.*, 1985; Mitchell *et al.*, 1987; Mitchell and Nairn, 1987; Mitchell, 1988a; Mitchell, 1988b), the UvrABC endonuclease assay detects only 55% repair after 8 hr (in the DFHR gene of CHO cells) and 66% after 24 hr post UV (Thomas *et al.*, 1989). These values are even lower in the downstream nontranscribed region, with only 33% and 27% of the (6-4) photoproducts removed by 8 and 24 hr, respectively. Although the authors indicate that this repair discrepancy may be due to the four-fold higher fluence used in their study (40 J/m^2) compared to Mitchell's work, the data presented here, which has been generated by enzymatic digestion of post-UV incubated human fibroblast DNA, indicates that this is not the case (see below). It therefore appears clear that the UvrABC technique is monitoring significantly more than (6-4) photoproducts and again signifies that a relative induction value of 66% is not correct. However, the value of 18% achieved in this present study is completely justifiable in light of the published data.

Within experimental error, the lesions removed from genomic DNA, as monitored by the enzymatic digestion / HPLC methodology, could be accounted for by their appearance in excision fragments. Along with the compatibility of induction rates obtained by this method with reported values, this lends immense support to this new protocol and furthermore implies that (i) excision fragments were not lost from the cell for the 24 hr under study [as has been previously reported (La Belle and Linn, 1982; Weinfeld *et al.*, 1986)]; (ii) recovery of trinucleotides during dialysis was complete; and (iii) the enzymatic digestion/HPLC methodology is an accurate means of enumerating the major UV photolesions. The accuracy of this method was further demonstrated by the consistency of kinetic data with that achieved using other techniques (see below).

Although both UV-damaged cellular constituents (DNA and excision fragments) are equally suited to this method, excision fragments have the advantage that there is a accumulation of lesions from zero upwards, whereas for DNA, one is looking at a sometimes small reduction from a very large number. This point is most obvious for the repair of T^T dimers (see Table III). Considering only the removal as seen in genomic DNA experiments, it might be surmised that repair did not begin until after 6 hr post UV. However, by examining excision fragments, it is obvious that these lesions were being repaired as early as 3 hr following irradiation. Thus, excision fragment analysis may be a more accurate means of quantitating damage repair by this protocol.

For ease of comparison, the cyclobutane dimer and (6-4) photoproduct repair data summarized in Tables III and V have been presented again in Figures 7 and 8, respectively. This detailed molecular analysis of both genomic DNA and the TCA-soluble material collected from post-UV incubated normal human fibroblasts reveals that cyclobutane dimers and (6-4) photoproducts were released with fundamentally different kinetics. The latter constituted the major excised lesions at 6 hr after UV-irradiation [~4.2 x 10^5 T[p]Py (6-4) photoproducts compared to ~1.1 x 10^5 T^T and T^C dimers]. Given that the number of C^C lesions repaired can be calculated as 0.24 x T^C (Weinfeld *et al.*, 1986), and C[p]C lesion repair as 0.27 x (T[p]T + T[p]C) [assuming that all (6-4) photoproducts are excised with the same proficiency; see page 25 for details], the total number of each class of UV lesions excised by this time was ~1.3 x 10^5 cyclobutane dimers and 5.3 x 10^5 (6-4) photoproducts. At sublethal fluences of UV light, this entails complete removal of the latter class as (i) the absolute number of (6-4) photoproducts appearing in excision fragments did not increases beyond this time (Table IV), and (ii) these photolesions were thereafter absent from the genomic DNA [certainly by 12 hr if not by 6 hr (see Table III)]. Cyclobutane dimers continued to accumulate, outnumbering the (6-4) photoproducts 2.5-fold by 24 hr post UV. However, only ~3.2 % of the UV-induced cyclobutane dimers had been excised by 3 hr post UV, reaching ~25% after a 24-hr repair period. In addition, both sets of experiments reveal that T^C dimers were repaired more rapidly than are T^T dimers, with nearly 50% of T^C lesions removed within 24 hr. This may be due to more efficient recognition by the repair complex. It is, for example, well documented that the cyclobutane dimer-specific enzyme, photolyase, is much more proficient at acting on T^T and T^C dimers than on C^C dimers (Myles et al., 1987).

The rapid removal of (6-4) photoproducts is in complete agreement with data by Mitchell and coworkers (1985; 1987) who, (as alluded to above) using a specific radioimmune assay, demonstrated that the loss of (6-4) photoproduct antibody binding sites was completed within 6 hr in UV-irradiated mammalian cells. Our additional findings, that (6-4) photoproducts can be recovered within short oligonucleotides in the TCA-soluble fraction, also confirms that these lesions are repaired by nucleotide excision repair, and not by pathways involving a (6-4) photoproduct-recognizing DNA glycosylase or a direct (6-4) photoproduct reversing enzyme (Friedberg, 1985). Recently, it has been suggested that (6-4) photoproducts may undergo changes to a Dewar isomer (Mitchell, 1988b; Taylor *et al.*, 1988) and that this photoproduct may constitute the ultimate cytotoxic lesion. Our collective data indicate that (6-4) photoproducts are excised without further transformation. Therefore, since the assay developed by Mitchell and coworkers is specific for the (6-4) photoproduct *per se*, and not the Dewar isomer, this implies that the loss of antibody binding sites truly reflects removal, rather than structural modification, of (6-4) photoproducts. This conclusion has recently been independently reported by others (Mitchell *et al.*, 1990a).

Within the first 6 hr following UV irradiation, cyclobutane dimers accounted for only ~20% of all excised lesions in a normal cell. Assuming that the repair of both classes of lesions induces a repair-patch of similar size, our results indicate that cyclobutane dimers would contribute to only ~20% of the total UDS at 6 hr after UV exposure, and (6-4) photoproducts would contribute the remaining 80%. Thus, the original assumption that UDS was measuring general repair is not true. At early times (≤ 6 hr), this repair assay is monitoring mainly (6-4) photoproduct repair, whereas by later repair times (24 hr) cyclobutane dimer removal is being observed. This has also been recently surmised by others (Mitchell and Nairn, 1989; Jones *et al.*, 1992). Given that UDS is classically measured at 2 hr post UV, it is not then surprising that UDS and UV endonuclease site removal (which is specific for cyclobutane dimer sites) often give conflicting results (Zelle and Lohman, 1979; Kraemer, 1983). [As will be illustrated in Chapter 6, TA* adducts were repaired with kinetics identical to that of (6-4) photoproducts and therefore also contribute to the 80% of total UDS at 6 hr. However, as removal of this minor class of UV photoproducts was indistinguishable from that of (6-4) photoproducts in all human fibroblast strains studied, their repair does not invalidate the above argument that UDS measured in the first 6 hr following UV irradiation is mainly of non-cyclobutane dimer origin.]

A further advantage to the enzymatic hydrolysis system used here is that it allows assessment of the status of the intralesion phosphodiester bond. Although reaction with nuclease P1 has been shown to give rise to a modified dimer (Liuzzi *et al.*, 1989), the conditions employed here (see Experimental Procedures) do not rupture the intradimer phosphodiester bond of a cyclobutane dimer. Extensive incubation (greater than 12 hr) at a pH of 5.5 is required to generate modified dimers with nuclease P1 (Liuzzi *et al.*, 1989). [(6-4) photoproducts are also not altered by this method (data not shown).] This is verified by the HPLC chromatogram shown in Figure 5B and 5C. Nuclease P1 digestion of the trinucleotides produced by enzymatic hydrolysis of UV-treated and unrepaired genomic DNA did not generate a species migrating at fraction 34, corresponding to dT<>dT modified cyclobutane dimers. Subsequent hot alkali treatment demonstrated that dT<>dC modified dimers were also not present. Cyclobutane dimers with severed intradimer phosphodiester bonds were, however, detected in excision fragments from post-UV incubated normal human fibroblasts. Enzymatic hydrolysis of these lesion-containing oligonucleotides generated dT<>dT and dT<>dC dimers, each of which had a distinct retention time on the reverse phase HPLC (see Figure 6), and whose identity could be confirmed by the generation of free labelled thymidine upon exposure to photochemical reversing fluences of UV light.

As is obvious from the data in Table IV, the vast majority of the cyclobutane dimers present in excision fragments isolated from post-UV incubated normal human fibroblasts were modified. Our results, therefore, conclusively demonstrate that cyclobutane dimers in excision fragments contained a cleaved intradimer phosphodiester bond, thereby confirming previous findings by an independent approach (Weinfeld *et al.*, 1986). Modified dimers were not present in genomic DNA after initial UV irradiation. The HPLC chromatograms obtained from post-UV incubated genomic DNA contained a very small percentage of total radiolabel at the retention times of modified dimers (data not shown). This would constitute, at most, 2% of the total number of cyclobutane dimers remaining in the genomic DNA at any given time. It was difficult to distinguish this small peak from background variation or from other coeluting compounds, such as thymine glycols. Furthermore, the dialysis procedure used to concentrate the lesion-containing trinucleotides would not be expected to retain completely modified dimer-containing trinucleotides. This would occur because after snake venom phosphodiesterase and calf alkaline phosphatase digestion, these trinucleotides would be left with only one internal phosphate. Such molecules would not be retained during dialysis as well as those with two internal charges (see Figure 2).

Although not distinguishable by the enzymatic digestion / HPLC assay system, modification of cyclobutane dimers most likely occurs prior to excision and is not merely a post-excision event. This is supported by radioimmune assays which estimate cyclobutane dimer excision. When removal of these lesions is followed by the disappearance of antibody-binding sites, repair appears to be more rapid than when measured by other protocols (Paterson *et al.*, 1984; Mitchell *et al.*, 1987; Roth *et al.*, 1987; Liuzzi *et al.*, 1989). This would be accounted for by dimer modification since severage of the intradimer phosphodiester bond would most likely remove antigenicity. Modified dimers have now been detected in post-UV incubated genomic DNA, using a much more sensitive technique. These occur only transiently and in specific regions of the genome (see Chapter 8).

It is notable that in a recent *in vitro* study discerning the points of incision relative to a cyclobutane dimer, modified cyclobutane dimers were not detected in the excised oligonucleotides (Huang *et al.*, 1992). In this investigation, incision was analyzed utilizing a human cell-free extract on a cyclobutane dimer-containing plasmid DNA. Following such treatment, a portion of the reaction mixture was deproteinized and subsequently subjected to photoenzymatic reversal. Contrary to the expectation if the cyclobutane dimers contained within the excised oligonucleotides had a severed interpyrimidine phosphodiester bond, this procedure did not generate a reduction in the size of the excised fragments as ascertained on a polyacrylamide gel. The authors additionally incubated a fraction of the photolyase-treated substrate with T4 DNA polymerase. The 3'→5' exonuclease activity of this prokaryotic polymerase, in the absence of deoxynucleotide triphosphates, has been shown to terminate at the 3' pyrimidine of both cyclobutane dimers and (6-4) photoproducts (Doetsch *et al.*, 1985). As the DNA polymerase was able to reduce completely the excised photolyase-treated oligonucleotides to mononucleosides, it can be concluded that photoenzymatic reversal had monomerized all of the cyclobutane dimers contained within the original excised fragment. This result implies that the excision fragments generated *in vitro* by cell-free extracts do not contain cyclobutane dimers with a severed interpyrimidine phosphodiester bond. However, this may not necessarily be true. Previous work, illustrating the existence of modified dimers in post-UV incubated XP group D cells (Paterson *et al.*, 1987), established that cyclobutane dimers, in which the intralesion phosphodiester bond has been cleaved, are a very poor substrate for photolyase. A substantial excess of the enzyme is therefore required to reveal these novel sites. However, if it were assumed that, in the study by Huang and coworkers (1992), photolyase treatment of the excised oligonucleotides was insufficient to remove a cyclobutyl ring connecting two pyrimidines lacking an internal phosphodiester bond, it would be predicted that the 3'→5' exonuclease activity of T4 polymerase would generate a truncated oligonucleotide with the pyrimidine dimer at the 3' terminus. However, it is not known how this exonuclease activity would respond to a modified lesion. In fact, for polymerases, we have predicted that they will bypass a cyclobutane dimer if and only if the intralesion phosphodiester bond has been severed. The same may be true of the 3'→5' exonuclease activity of T4 polymerase. Therefore the inability of photolyase to expose a severed intradimer phosphodiester bond, or the inability of T4 polymerase to reveal a lesion which has not been monomerized by photolyase, cannot be taken as conclusive evidence that the cyclobutane dimers contained in the excised oligonucleotides lacked this modification.

Regardless of the above argument, the preferred hypothesis addressing the apparent lack of modified dimers in the lesion-containing oligonucleotides excised by a human cell-free extract, is that this modification is not a prerequisite to (as has been hypothesized by Paterson *et al.*, 1987) or directly associated with nucleotide excision repair. They are, therefore, quite likely not generated in this *in vitro* reaction. In fact, given the *in vitro* reaction conditions under which nucleotide excision is assayed, it can be surmised that IDP modification of a cyclobutane dimer is not a necessary preincision event. The human IDP activity isolated in our laboratory has a pH optimum of 5.0, with no activity above pH 7.0. However, *in vitro* incision assays are performed at pH 7.5, and repair synthesis assays at 7.9 (Ullah *et al.*, 1989). However, the premise put forth in this dissertation is that intradimer phosphodiesterase acts on a cyclobutane dimer in preparation for, or as an intimate part of, replication and/or transcription. Therefore, in the *in vitro* nucleotide excision repair study of Huang *et al.* (1992), a lack of S-phase replicational and transcriptional activity would account for the absence of modified cyclobutane dimers.

Although cyclobutane dimers were often found in excision fragments with a cleaved intradimer phosphodiester bond, (6-4) photoproducts were always excised with this bond still intact (see Figure 9). This may, in part, explain the difference in removal kinetics of the two lesion types. If (6-4) photoproducts are indeed the major cytotoxic lesion as has been suggested (Clarkson *et al.*, 1983; Mitchell *et al.*, 1987; Mitchell *et al.*, 1988; Mitchell, 1988a), their rapid removal may be essential to cell survival. However, it is known that cyclobutane dimers block the progression of both prokaryotic and eukaryotic DNA and RNA polymerases (Caillet-Fauquet *et al.*, 1977; Villani, G., *et al.* 1978; Moore *et al.*, 1981; Vos and Rommelaere, 1982; Doetsch *et al.*, 1985; Griffiths and Ling, 1989; Selby and Sancar, 1990) and thus they would be expected to be equally cytotoxic. However, modification may alleviate this block, and thereby buy time until these lesions can be repaired. The ability to overcome barriers to replication is not an unprecedented hypothesis (Vos and Rommelaere, 1982). This speculation is dealt with in greater detail in Chapter 8.

Although it is known that cyclobutane dimers are repaired in human cells by the nucleotide excision mode of repair, there has been little information until very recently as to the size of the excision fragments themselves. Using an *in vitro* human nucleotide excision repair system, it has been concluded that UV-damaged DNA is incised 21 -23 nucleotides upstream and 5 - 6 nucleotides downstream of a cyclobutane dimer (Huang et al., 1992). The data presented in this chapter imply that lesions are excised in oligonucleotides of 7 nucleotides in length. This is surprising in light of this most recent *in vitro* work, and considering that *E. coli* has excision fragments of 12 to 13 nucleotides (Yeung *et al.*, 1983; Sancar and Rupp, 1983). Although this work is not definitive, several points would suggest that the small oligonucleotides studied here are not degradation products of the original excision fragments. Firstly, if excision fragments were being degraded in the cell, one would expect to see a distribution of sizes. However, by far the major length fragment is a heptamer. Secondly, if these were smaller versions of the original excised single-stranded DNA molecules, it would be expected that some of the lesions would be "missing" in this assay as longer fragments would not be collected in their entirety because of their TCA solubility. Finally, fragment length does not change with extended repair time. Thus, the fragments excised as 7-mers at 3 hr post UV remain as 7-mers by 24 hr. To absolve this result with the work of Huang *et al.* (1992), it is possible that *in vitro* nucleotide excision repair does not precisely mimic *in vivo* mechanistics. Alternatively, a 7-mer may be produced by action of a 5'→3' exonuclease, whose progression is blocked by the lesion, acting on the longer 27 - 29 nucleotide excision oligonucleotides. Such an exonuclease would need to act immediately following excision as there is no difficulty in recovering 100% of the excised lesions in the TCA soluble fraction of post-UV incubated cells, even though oligonucleotides as small as 10 nucleotides have limited TCA solubility.

There may exist more than one repair mechanism operating on UV-induced damage in human cells. The two major classes of lesions are repaired with very different kinetics (Mitchell *et al.*, 1985; Mitchell and Nairn, 1989; this study, Figures 7 and 8). In addition, the majority of cyclobutane dimers found in excision fragments have a cleaved intradimer phosphodiester linkage (Weinfeld *et al.*, 1986; this study, Table IV). The (6-4) photoproducts, on the other hand, appear to always contain an intact phosphodiester bond. Although both classes of lesions are removed by nucleotide excision repair, it is quite likely that at least the initial recognition and/or incision steps for these two lesions are different.

It has recently been suggested that the different rates of excision for the two major classes of UV-induced DNA damage reflect a preferential recognition of the same incision complex for (6-4) photoproducts. These lesions would, therefore, compete more successfully for some limiting factor of nucleotide excision repair (Jones *et al.*, 1992). This not only offers an explanation for this difference in kinetics, but, in addition, can account for UV-sensitive cell strains which remain competent for (6-4) photoproduct repair while partially, or totally, deficient at cyclobutane dimer removal. However, this proposal does not readily account for those strains which are proficient at cyclobutane dimer repair, but lacking in (6-4) photoproduct excision (see Chapter 4). Therefore, the hypothesis that two separate recognition/incision mechanisms are operating is not unwarranted.

In conclusion, the three-stage procedure used to analyze genomic DNA or excision fragments derived from post-UV incubated cells, has proven useful for the measurement of repair of (6-4) photoproducts and intact or modified cyclobutane dimers in normal human fibroblasts. As will be presented in Chapters 3 through 6, this methodology can be extended to other normal or repair-deficient mammalian cells, such as fibroblasts strains derived from xeroderma pigmentosum patients (Kraemer, 1983) and Chinese hamster ovary cells (Thompson *et al.*, 1981), and to the study of another minor photoproduct, the TA* adduct. In addition to the analysis of UV-induced DNA damage, this novel methodology should also lend itself to the examination of other DNA adducts that are repaired via nucleotide excision repair (Paterson *et al.*, 1984).

repair time (hr)	dpm before dialysis	dpm after dialysis	% dpm retained
0	18,815,600	90,100	0.479
3	16,728,000	85,900	0.513
6	16,910,000	85,700	0.507
12	19,515,000	93,100	0.477
24	17,537,000	77,800	0.444
sham-irradiated	18,215,000	65,000	0.357

TABLE I Radioactivity retained after dialysis of hydrolysed UV-irradiated (40 J/m^2) human (GM38) genomic DNA. Samples were dialyzed in 3 kDa MW cut-off tubing against ddH$_2$0 for 24 hr.

repair time (hr)	% dpm in d-T<p>C	% dpm in dT<>dC	% dpm in d-T<p>T	% dpm in dT<>dT	% dpm in d-T[p]Py
0	7.9	0.2	21.8	0.1	3.3
3	7.2	0.2	20.3	0.1	1.9
6	7.2	0.3	20.6	0.1	0.9
12	6.8	0.3	20.9	0.1	0.6
24	4.8	0.2	20.7	0.0	0.5
sham-irradiated	0.00	0.3	0.34	0.1	0.6

TABLE II Percentage of radioactivity in lesion peaks after nuclease P1 digestion and HPLC analysis of labelled trinucleotides (generated by enzymatic digestion of genomic DNA and enriched by dilaysis) derived from post-UV (40 J/m^2) incubated normal human fibroblasts.

repair time (hr)	d-T<p>C $\times 10^{-6}$	d-T<p>T $\times 10^{-6}$	d-T[p]Py $\times 10^{-6}$
0	1.21	1.66	0.50
3	1.19	1.66	0.31
6	1.16	1.66	0.14
12	1.03	1.59	0.08
24	0.68	1.46	0.07
sham irradiated	0.00	0.02	0.07

TABLE III UV photoproducts remaining in genomic DNA of a single cell following post-UV (40 J/m^2) incubation of normal human fibroblasts. Calculations are based on the following assumptions: (i) in an asynchronous population, each human diploid fibroblast has 6 x 10^{-12} g (i.e. 3.6 x 10^{12} Da) of genomic DNA (Sober, 1968); (ii) assuming the average molecular weight of a mononucleotide to be 327 Da, there are therefore 1.10 x 10^{10} nucleotides per cell; (iii) in human DNA, the fraction of nucleotides containing thymine is 0.29 (Sober, 1968); hence the number of thymine residues per genome equals 3.19 x 10^9; (iv) the percentage of radiolabel is divided by two in order to compute T^T since either Thy can be labelled; (v) the vast majority of thymidine-containing (6-4) photoproducts are T[p]C (Lippke *et al.*, 1981; Brash and Haseltine, 1982; Franklin *et al.*, 1982) and therefore no correction has been made for T[p]T.

UV fluence (J/m^2)	time (hr)	% [^3H] Thy in major peaks													
		formic acid hydrolysis			enzymatic hydrolysis					alkaline hydrolysis					
		T<>U	T<>T	Thy	d-T<p>C	d-T<p>T	dT<>dC d-T[p]Py	dT<>dT	dT	T[]Py	d-T<p>C	d-T<p>T	dT<>dC	dT<>dT	dT
40	0	0.0	0.0	93.3	0.0	0.0	0.0	0.0	92.4	0.0	0.0	0.0	0.0	0.0	92.1
40	3	1.2	3.0	82.0	0.9	1.7	11.5	3.3	65.3	11.7	1.2	1.7	1.4	3.0	64.8
40	6	4.1	4.2	75.8	4.0	3.3	23.3	5.9	50.4	25.2	3.9	3.5	1.2	5.6	52.1
40	12	7.9	9.6	69.7	4.2	3.1	17.9	8.3	55.3	15.3	5.3	3.3	3.9	8.0	54.7
40	24	13.6	23.0	56.0	4.5	4.1	19.3	17.1	43.4	10.1	4.4	3.6	9.2	17.3	43.2
0	24	0.0	0.0	87.8	0.0	0.0	0.0	0.0	90.8	0.0	0.0	0.0	0.0	0.0	89.7

TABLE IV Distribution of hydrolysed excision fragments from post-UV incubated (40 J/m^2) normal human fibroblasts into various species separated by reverse-phase HPLC. NOTE: The percentages of total counts indicated in the table for each assay do not add up to 100% because a minor fraction of radiolabel is distributed over the remainder of each chromatogram.

UV fluence (J/m^2)	repair time (h)	[^{3}H]Thy (dpm/dish)		%Thy in acid soluble [A/B]= [C]	% Thy involved in UV-lesions in acid-soluble			% of total Thy in excised UV-lesions (x10^3)			UV-induced lesions excised per cell (x10^4)		
		acid soluble [A]	total x10^4 [B]		[a] T^T [D][c]	[a] T^C [E][c]	[b] 6-4 [F][d]	T^T [CxD] =[G]	T^C [CxE] =[H]	6-4 [CxF] =[I]	T^T [GxJ] x0.5	T^C [HxJ]	[f] 6-4 [IxJ] [g]
40	0	9700	32.9	0.029	0.0	0.0	0.0	0.0	0.0	0.0	0	0	0
40	3	13,500	32.8	0.041	3.0	1.2	10.1	1.2	0.5	4.1	2	2	13
40	6	17,950	31.5	0.057	4.2	4.1	23.2	2.4	2.3	13.2	4	7	42
40	12	30,950	33.3	0.093	9.6	7.9	14.2	8.9	7.3	13.2	14	23	42
40	24	39,425	33.0	0.119	23.0	13.6	10.2	27.4	16.2	12.2	44	52	39
0	24	8900	32.1	0.028	0.0	0.0	0.0	0.0	0.0	0.0	0	0	0

TABLE V The accumulation of UV lesion-containing excision fragments in post-UV (40 J/m^2) incubated normal human fibroblasts (GM38). [a]T^T and T^C refer to all thymine-thymine and thymine-cytosine containing cyclobutane dimers, respectively. [b]6-4 refers to T[p]Py (6-4) photoproducts only. [c]The % of Thy dimerized in the acid-soluble fraction is based on formic acid hydrolysis. [d]The % of Thy involved in (6-4) photoproducts in the acid-soluble fraction is calculated as [% counts in dT<>dC and d-T[p]Py + % counts in d-T<p>C - % counts in T<>U (from Table IV)]. [e]Calculations are based on the same assumptions set forth in Table III, and therefore J (the number of thymidine residues per human genome) equals 3.19 x 10^9. It is also assumed that all excised UV lesions remain inside the cell and are recovered in the acid-soluble material. [f]See Weinfeld *et al.*, 1986. [g]By far the major thymine-containing (6-4) photoproduct is T[p]C (Lippke *et al.*, 1981; Brash and Haseltine, 1982; Franklin *et al.*, 1982)) and therefore no correction has been made for T[p]T.

treatment	% recovery of [^{3}H]-labelled DNA	
	UV-irradiated $p(dT)_{10}$	unirradiated $p(dT)_{10}$
5% TCA	52	28
5% TCA + 2 mg/ml BSA	86	81
5% TCA + 5 x 10^6 CELLS	72	70

TABLE VI Solubility of radiolabelled oligonucleotides in conditions used to isolate excision fragments.

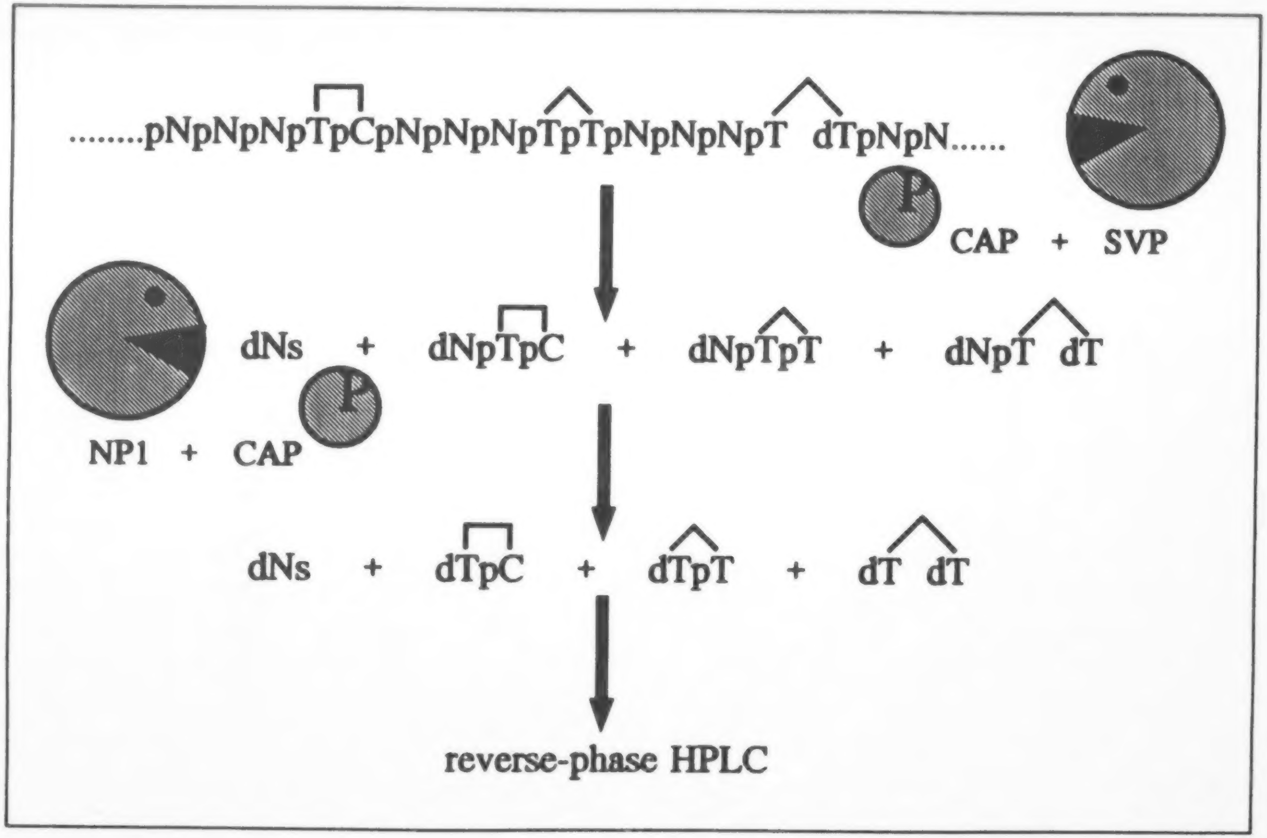

.........pNpNpNpTpCpNpNpNpTpTpNpNpNpT dTpNpN......
CAP + SVP
dNs + dNpTpC + dNpTpT + dNpT dT
NP1 + CAP
dNs + dTpC + dTpT + dT dT
reverse-phase HPLC

FIGURE 1 Illustration depicting the enzymatic hydrolysis / HPLC assay for quantitating UV photoproducts in [^{3}H] thymidine-labelled DNA.

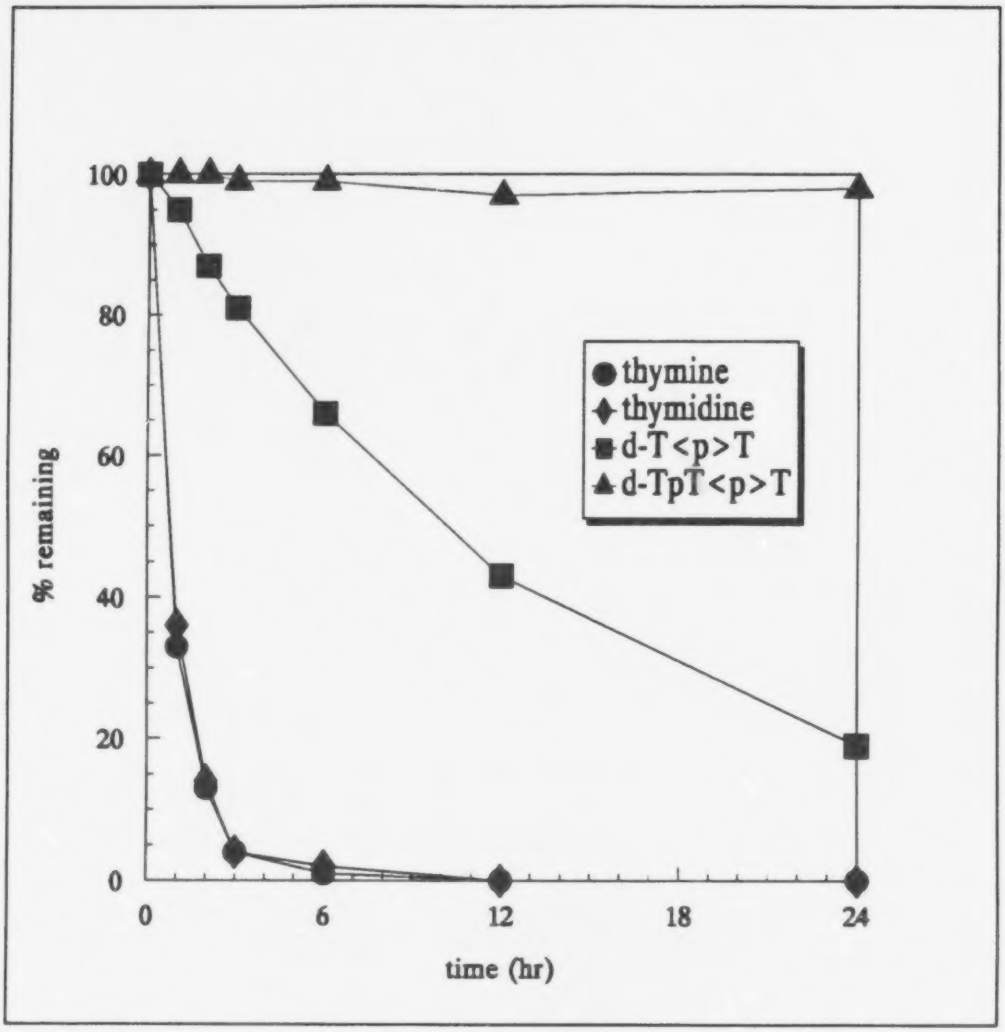


FIGURE 2 Retention, in dialysis tubing, of small nucleic acid species as a function of dialysis time.

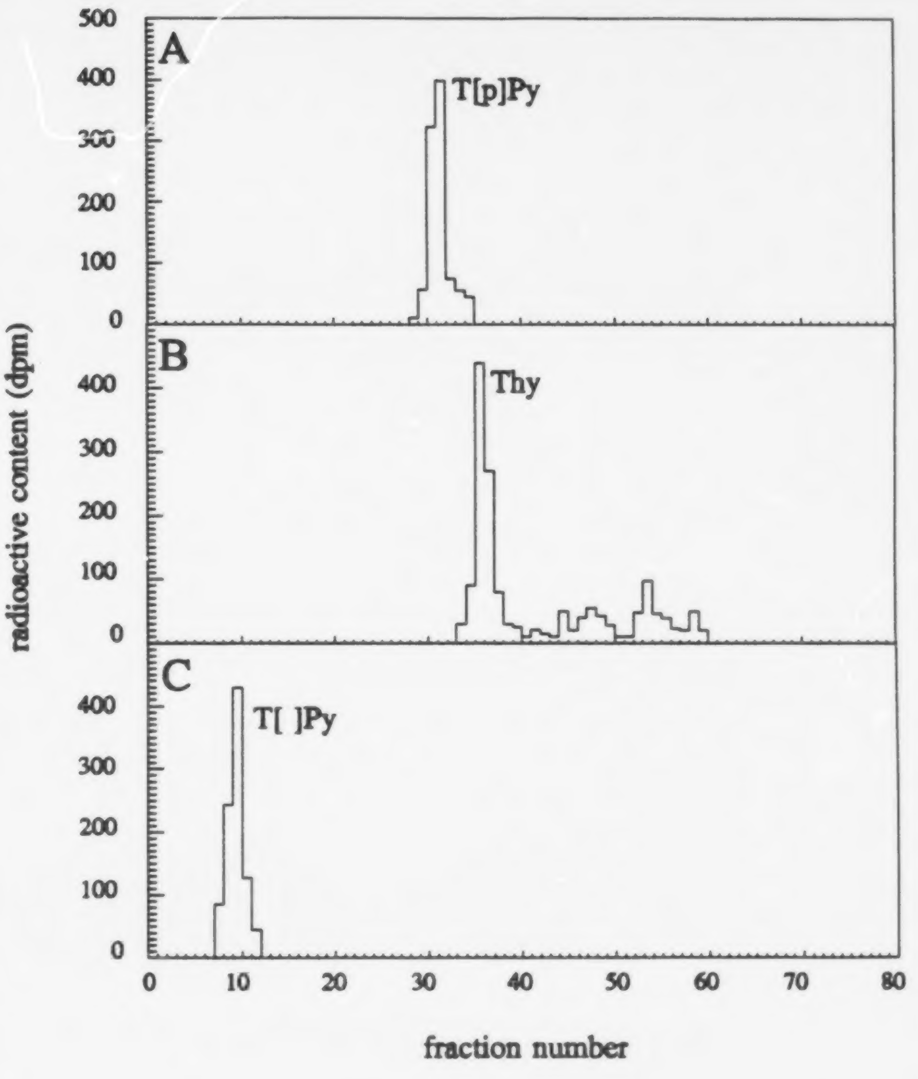


FIGURE 3 Reverse-phase HPLC radioactivity profiles of the material eluting at fractions 28-34 from reverse-phase HPLC after enzymatic digestion of [^{3}H] thymidine-labelled UV-treated DNA. Reinjection after: A. purification as indicated in Experimental Procedures; B. formic acid hydrolysis; and C. hot alkaline treatment.

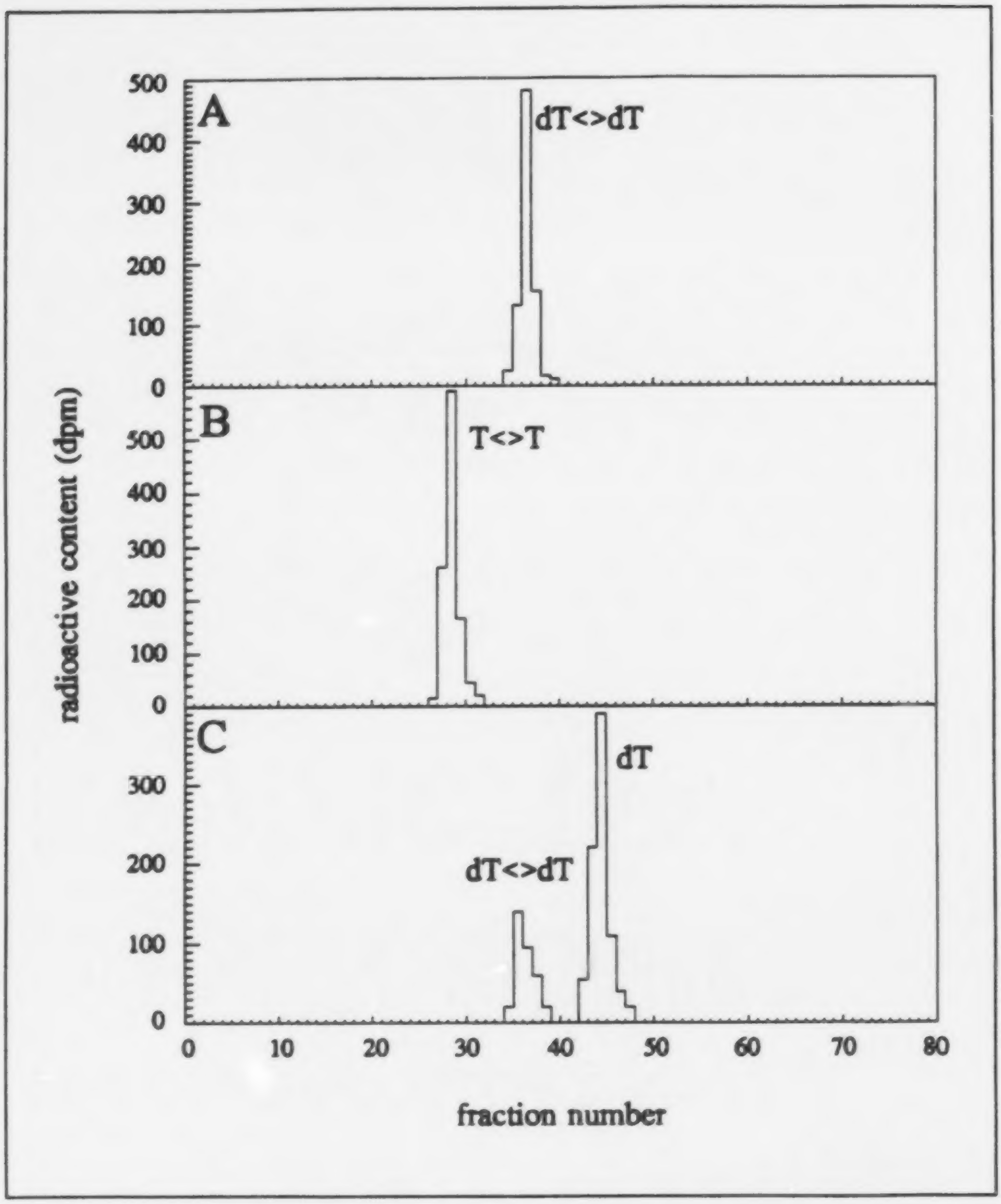


FIGURE 4 Reverse-phase HPLC radioactivity profiles of the material eluting at fractions 34-37 on reverse-phase HPLC after enzymatic digestion of [^{3}H] thymidine-labelled UV-treated DNA. Reinjection after: **A.** purification as indicated in Experimental Procedures; **B.** formic acid hydrolysis; and C. photochemical reversal (5500 J/m^2 of 254-nm light).

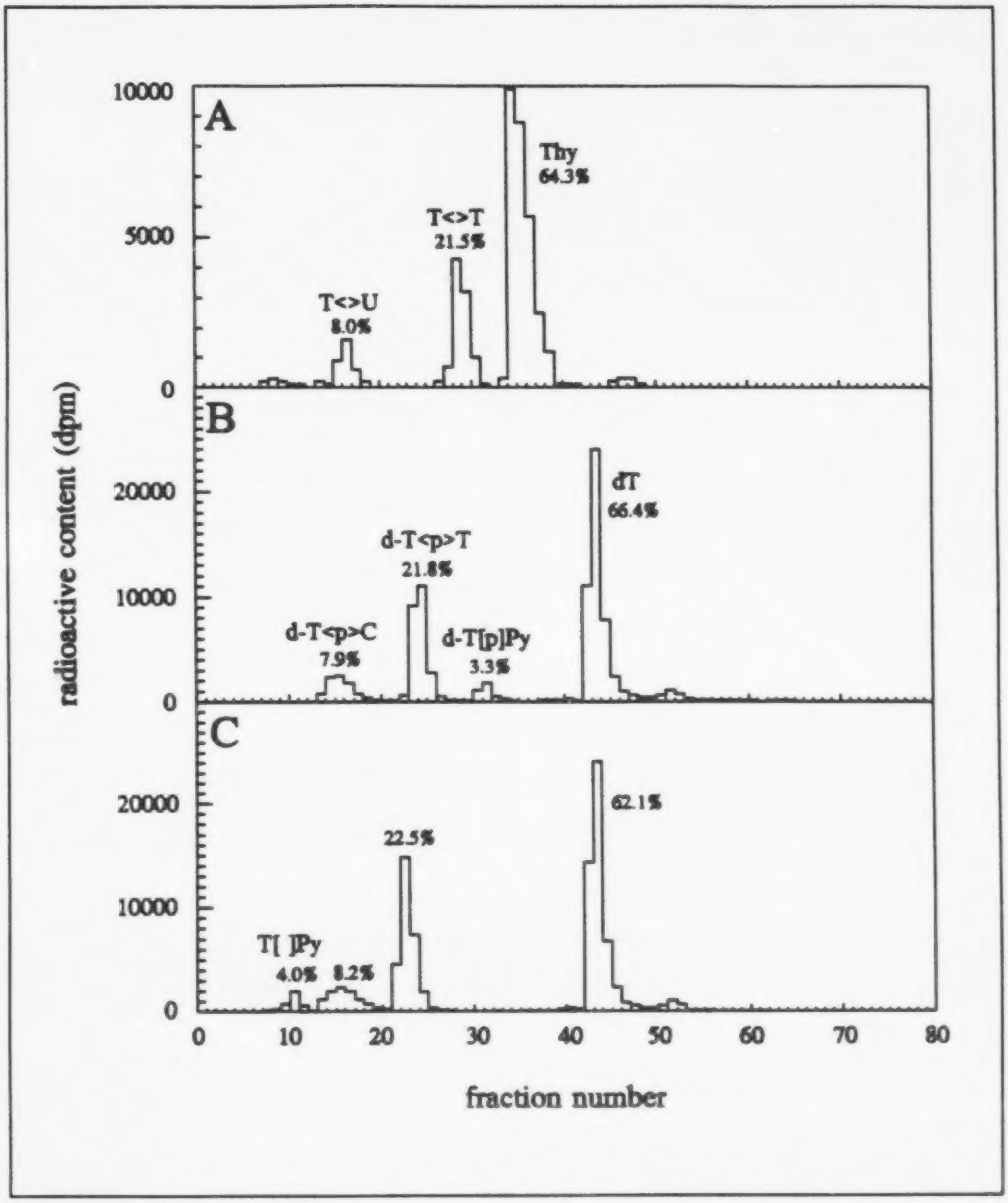


FIGURE 5 Reverse-phase HPLC radioactivity profiles of partially purified trinucleotides obtained from UV-treated (40 J/m^2) normal human fibroblast (GM38) DNA. **A.** Formic acid hydrolysis; **B.** Enzymatic hydrolysis; and **C.** Hot alkaline treatment. Numbers indicated on chromatograms refer to % of total radioactivity in each major peak.

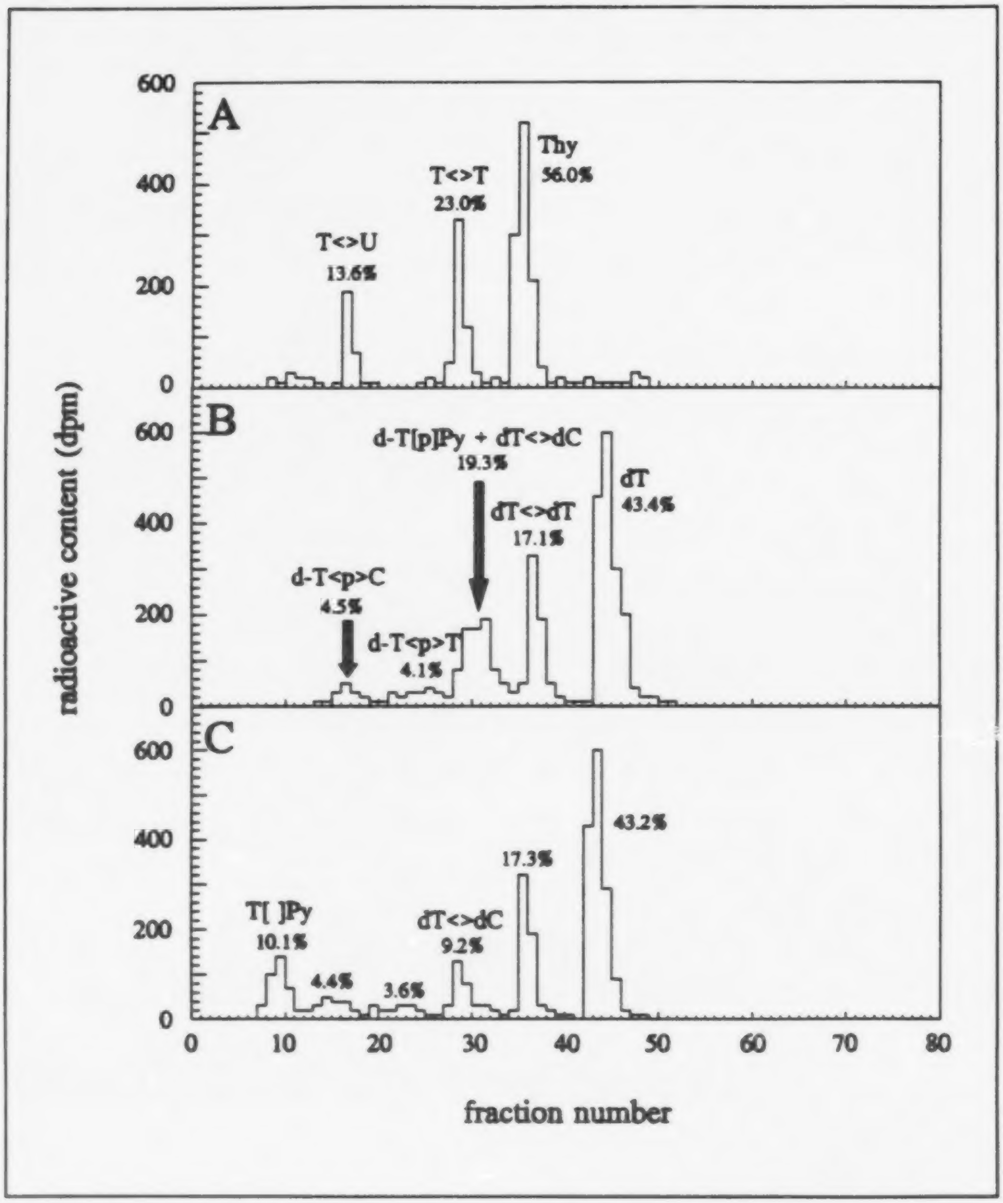


FIGURE 6 Reverse-phase HPLC radioactivity profiles of excision fragments isolated from post-UV (40 J/m^2) incubated (24 h) normal human fibroblasts (GM38). **A.** Formic acid hydrolysis; **B.** Enzymatic hydrolysis; and C. Hot alkaline treatment. Numbers indicted on chromatograms refer to % of total radioactivity in each major peak.

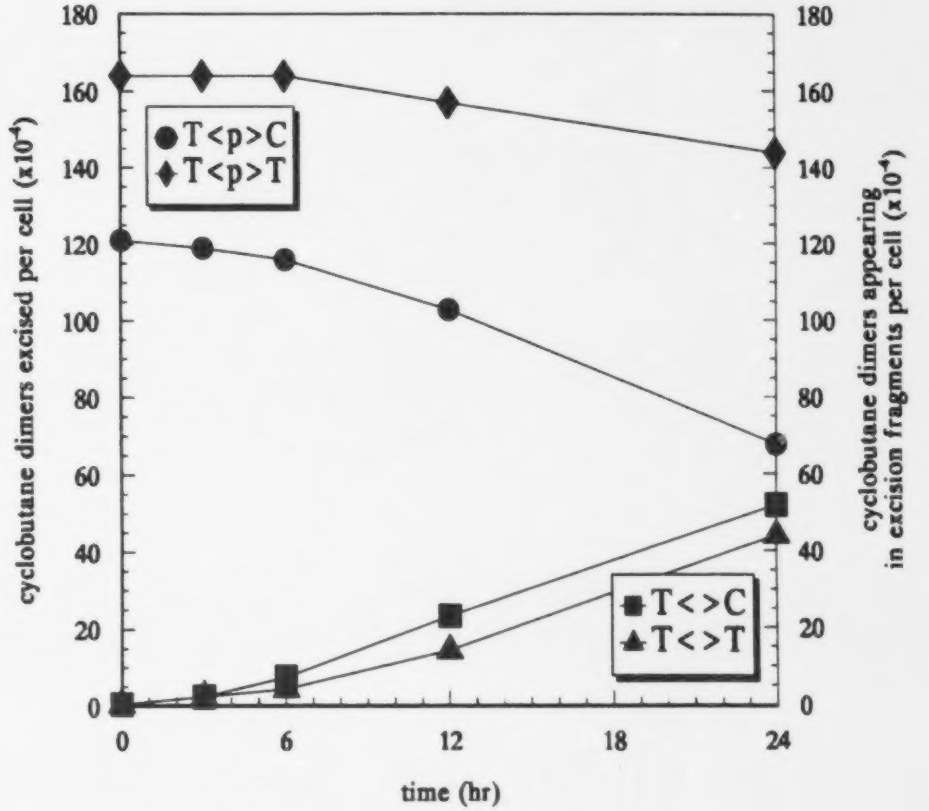


FIGURE 7 Repair of cyclobutyl dimers, following 40 J/m^2 of 254-nm light, in normal human fibroblasts (GM38). Diamonds and circles show the removal of T^T and T^C lesions, respectively, from genomic DNA. Squares and triangles illustrate the accumulation of T^T and T^C dimers, respectively, in excision fragments without regard to the status of the intradimer phosphodiester bond.

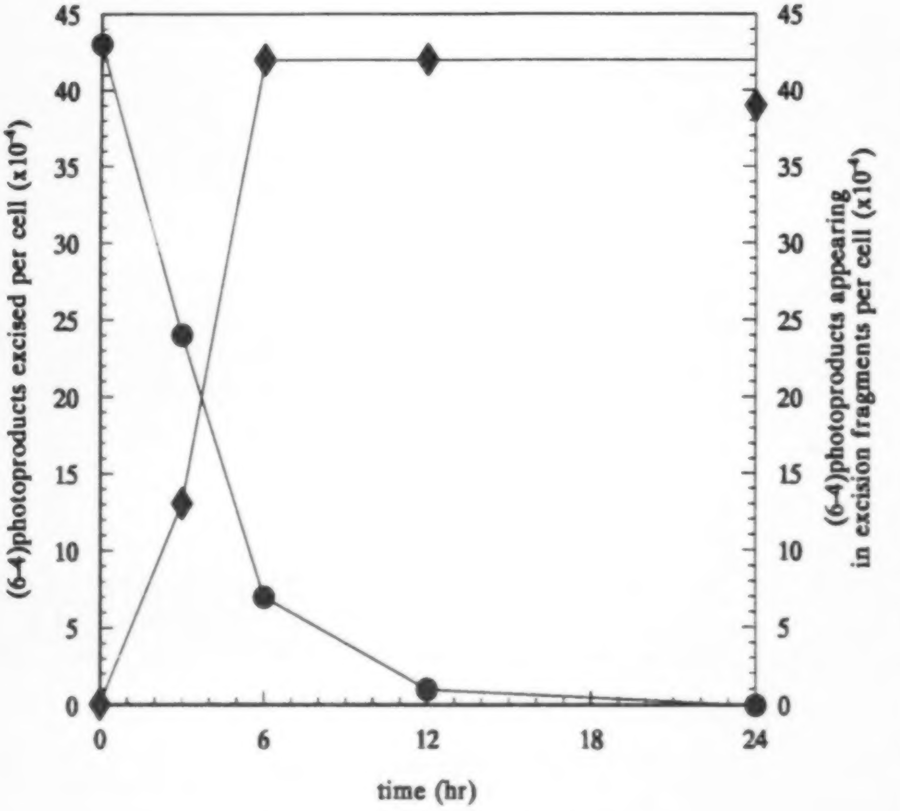


FIGURE 8 Repair of T[p]Py (6-4) photoproducts, following 40 J/m^2 of 254-nm light, in normal human fibroblasts (GM38). Circles show the removal of these lesions from genomic DNA, and diamonds illustrate their accumulation in excision fragments.

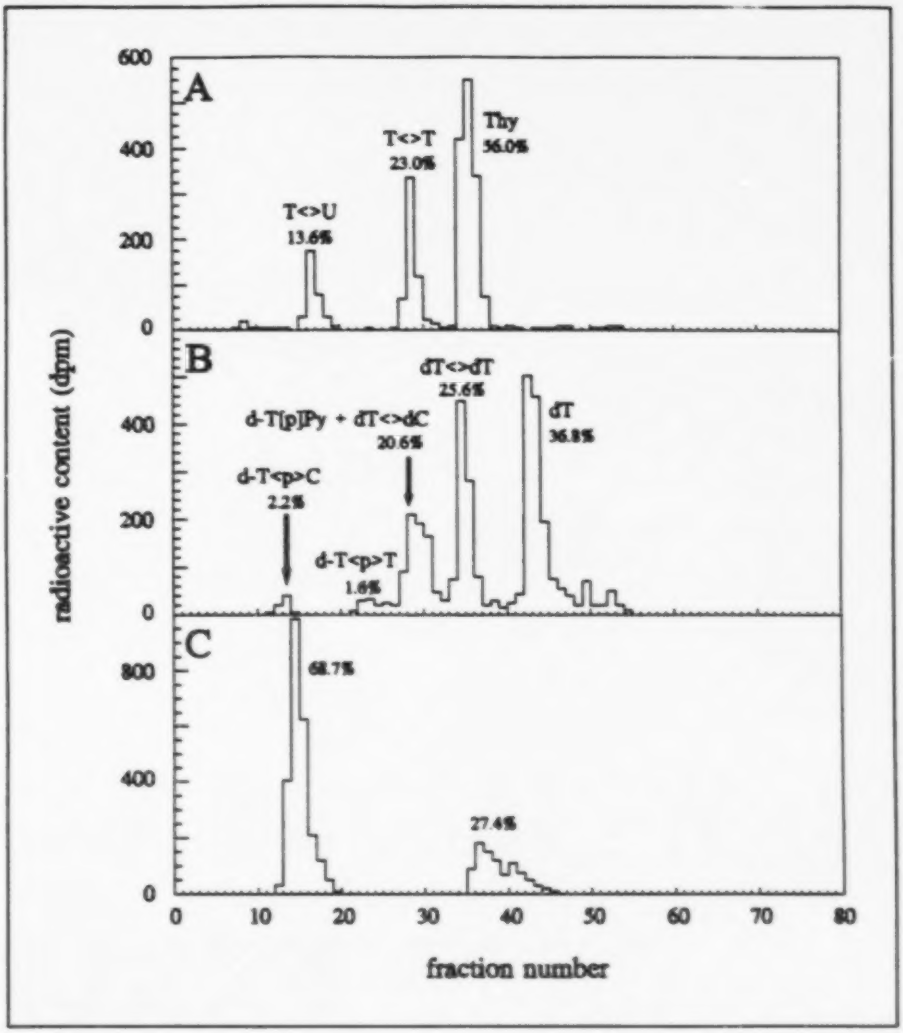


FIGURE 9 HPLC radioactivity profiles of excision fragments isolated from post-UV (40 J/m^2) incubated (24 hr) normal human fibroblasts (GM38). **A.** Formic acid hydrolysis, followed by reverse-phase chromatography. **B.** Enzymatic hydrolysis, followed by reverse phase chromatography. **C.** Enzymatic hydrolysis, followed by SAX chromatography. Numbers indicated on chromatograms refer to % of total radioactivity in each major peak. The uncharged molecules generated by enzymatic hydrolysis [if (6-4) photoproducts have an intact intralesion phosphodiester bond] are dT<>dC, dT<>dT, and dT. The % dT<>dC is calculated as the % T<>U following formic acid hydrolysis (13.6%) - the % d-T<p>C after enzymatic hydrolysis (2.2) = 11.4%. Therefore, the % uncharged molecules expected = 11.4 + 25.6 + 36.8 = 73.8%.

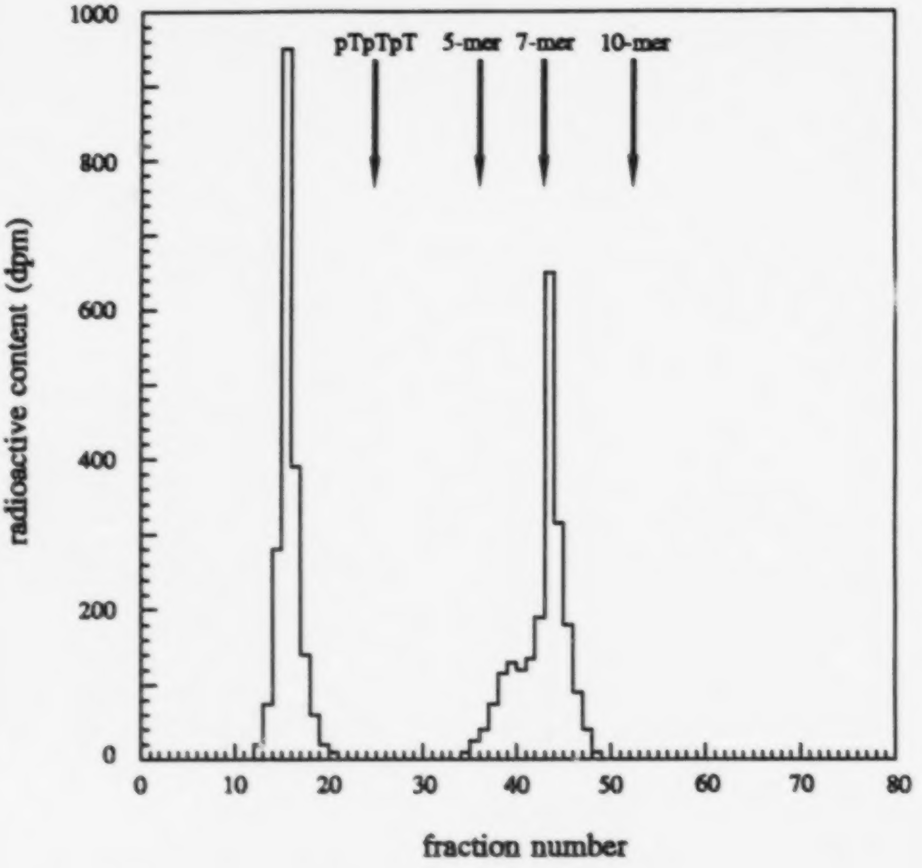


FIGURE 10 SAX profile of excision fragments isolated from post-UV (40 J/m^2) incubated (12 hr) normal human fibroblasts (GM38). Number markers indicate the elution time of various length oligonucleotides.

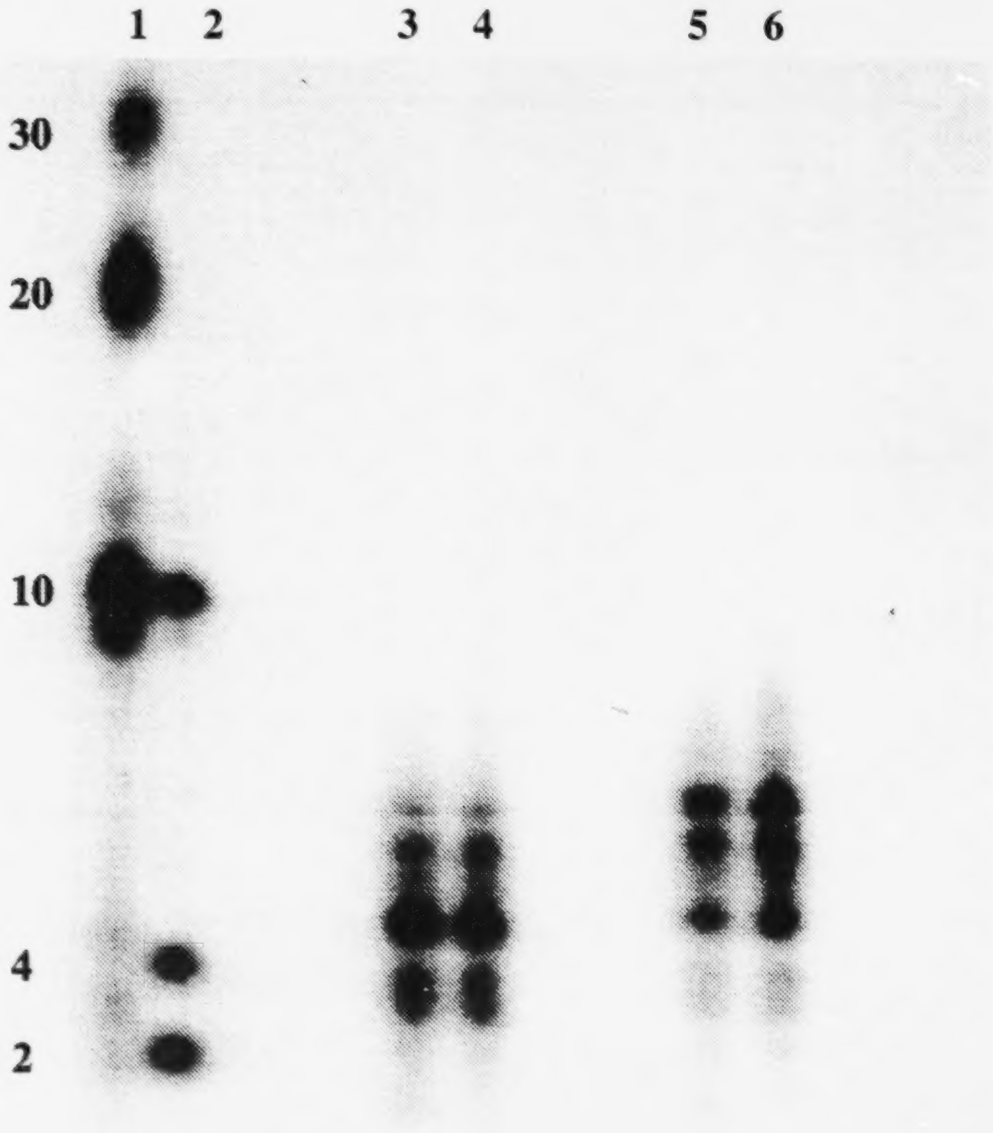


FIGURE 11 Polyacrylamide gel illustrating the lengths of oligonucleotides contained in the TCA-soluble fraction derived from post-UV (40 J/m^2) incubated (12 hr) normal human fibroblasts (GM38). Oligonucleotides have been [^{32}P] end-labelled prior to electrophoresis. Lanes 1 and 2: marker oligonucleotides; Lanes 3 and 4: kinase reaction #1 of the TCA-soluble material; Lanes 5 and 6: kinase reaction #2 of the same TCA-soluble material. The lengths of marker oligonucleotides are indicated.

CHAPTER THREE - REPAIR OF UV PHOTOPRODUCTS IN CHINESE HAMSTER OVARY CELLS: AN EXPLANATION FOR THEIR HIGH UV SURVIVAL

INTRODUCTION

The development of a procedure involving enzymatic hydrolysis of "post-UV incubated genomic DNA" or of the DNA fragments excised during post-UV incubation (i.e. excision fragments), offers a novel approach for assessing a cell's ability to repair UV-induced DNA damage (Chapter 2, Figure 1, page 47). This technique has been used to examine UV repair in Chinese hamster ovary cells. These cells have been the subject of much study recently as their relatively high UV-survival, given their apparent inability to remove cyclobutane dimers, has been a longstanding dilemma (Trosko *et al.*, 1965; Klímek, 1966; Vijg *et al.*, 1984). CHO cells are now known to excise efficiently a second minor class of photoproduct, (6-4) photoproducts (Mitchell *et al.*, 1985), and display preferential repair of transcriptionally active sequences (Bohr *et al.*, 1985).

Excision fragment studies have classically been unable to detect cyclobutane dimer repair in CHO cells (Trosko and Kasschau, 1967). This is likely because these cells contain very large nucleotide pools (Yagi *et al.*, 1984; this study). As a result, the lesion-containing excision fragments represent a minority of the radiolabelled TCA-soluble material. In addition, their limited ability to repair cyclobutane dimers makes detection of repair by studying genomic DNA virtually impossible (Yagi *et al.*, 1984). Nonetheless, using our current methodology with either genomic DNA or excision fragments, it has been possible to confirm the previous report regarding the proficiency with which CHO cells repair (6-4) photoproducts (Mitchell *et al.*, 1985). Under the irradiation conditions employed here (40 J/m^2 of 254-nm light), all (6-4) photoproducts induced in the DNA are removed within 6 hr. Furthermore, within 24 hr, CHO cells repair less than 4% of the cyclobutane dimers initially present under these irradiation conditions.

As is the case for cultured human fibroblasts, (6-4) photoproducts are recovered in excision fragments from CHO cells with an intact internal phosphodiester linkage. Although very few cyclobutane dimers are removed during post-UV incubation in these rodent cells, the majority of such lesions that are found in excision fragments have a cleaved intradimer phosphodiester bond. Thus, as is true for human fibroblasts, these cells appear to possess an intradimer DNA phosphodiester activity specific for cyclobutane dimers (Weinfeld *et al.*, 1986; Liuzzi and

Paterson, in preparation). Therefore, with the exception of the extent of cyclobutane dimers removal (normal human cells remove ~25% of cyclobutane dimers induced under the same conditions that CHO cells repair 4%), repair characteristics are indistinguishable from those observed in human cells.

As expected from their UV-sensitive phenotypes, the two CHO mutants examined in this study, UV-4 and UV-5, are no longer capable of repairing (6-4) photoproducts (in agreement with Mitchell *et al.*, 1988), nor do they repair the small number of cyclobutane dimers repaired by the parent cell line, AA8. These data, therefore, give no information as to which lesion [cyclobutane dimers or (6-4) photoproducts] causes the increased UV-sensitivity in these mutant lines. The loss of cyclobutane dimer repair in UV-4 and UV-5 is in contrast to Mitchell *et al.* (1988) and Regan *et al.* (1990), who showed by radioimmunoassay and by a two-dimensional radiochromatographic assay, respectively, that cyclobutane dimer excision did not differ in these lines from the parent strain. However, the former assay indicates a much higher excision rate for cyclobutane dimers in normal human cells than has been demonstrated by any other method (Williams and Cleaver, 1979; Mitchell *et al.*, 1982; Vijg *et al.*, 1984; Mitchell *et al.*, 1985). The authors, themselves, suggest that this higher apparent repair in normal human cells may be due to dimer modification (Mitchell *et al.*, 1988). It is therefore possible that what has appeared in these earlier studies to be dimer removal in these CHO UV-sensitive mutants is actually dimer modification. This, along with proficient (6-4) photoproduct repair and preferential repair of cyclobutane dimers from transcriptionally active genes (Bohr *et al.*, 1985), is addressed, in the Discussion of this chapter, in relation to the high UV survival of rodent cells.

Given that CHO and human cells share many repair capabilities, a normal repair gene from one species should substitute for a defective (or absent) gene in the other in a manner grossly similar to the complementation of several xeroderma pigmentosum (XP) and CHO mutant strains with the T4 *denV* gene (Valerie *et al.*, 1985; Valerie *et al.*, 1987). It is therefore not surprising that several human repair genes have been isolated utilizing repair-deficient CHO cells as recipients of normal human DNA in DNA-mediated gene transfer experiments, e.g., *ERCC-1* (Rubin *et al.*, 1983; Westerveld *et al.*, 1984), *ERCC-2* Weber *et al.*, 1988; Weber *et al.*, 1990), *ERCC-3* (Weeda *et al.*, 1990a), *ERCC-5* (Cox and Gedde-Dahl, 1985; Thompson *et al.*, 1987), and ERCC-6 (Hoeijmakers *et al.*, 1989; Troelstra *et al.*, 1990).

EXPERIMENTAL PROCEDURES

For more detailed experimental procedures, see Appendix A, page 254.

Cell strains. The following four diploid strains were utilized for one or more of the experiments outlined below: (i) GM38 (described in Chapter 2), (ii) AA8, a normal Chinese hamster ovary cell line, and (iii) UV-4, and (iv) UV-5, both derived from AA8 by EMS mutagenesis and selected for on the basis of their increased sensitivity to UV irradiation (Thompson *et al.*, 1980). All three CHO cell strains were gifts from Dr. Larry H. Thompson (Lawrence Livermore Laboratory, Livermore, CA). Much of the data presented here for GM38 have been taken from Chapter 2 so as to facilitate direct comparison to CHO cells.

Material, culture conditions and radioactive labelling of cellular DNA. Materials and methods were the same as those described in Experimental Procedures of the preceding chapter (see page 14) with the following exception. As CHO cell lines appear to be particularly sensitive to radioactive labelling of their DNA, cultures were incubated in [^{3}H]thymidine-containing medium for only 24 hr, instead of 48. This was found to have the desired effect of maintaining high incorporation of label into the genomic DNA while reducing cell death.

UV irradiation, subsequent incubation of cell cultures, and isolation of trinucleotides derived from enzymatic digestion of genomic DNA. The removal of lesions from genomic DNA was examined only in the case of the parent cell line, AA8. Procedures were the same as those in Chapter 2 (see page 15, 258) except that repair times included 1, 2, 3, 4, 6, 12, and 24 hr post UV.

Isolation and SAX analysis of excision fragments. Excision fragments were isolated as the TCA-soluble fraction from post-UV incubated CHO cells as described in Chapter 2 (see page 16) for human cells. However, direct analysis of this material, as had been done previously for that derived from human cells, proved difficult. As the initial examination of such material displayed an extremely low yield of all UV photoproducts, SAX-HPLC was performed to determine the size of the [^{3}H]-labelled species present. SAX analysis, using the method outlined in Chapter 2 (see page 18), was conducted on the TCA-soluble material from normal CHO cells (AA8), as well that isolated from post-UV incubated normal human fibroblasts (GM38). The retention times of specific molecules, namely thymidine, TMP, TDP, and TTP, were identified using authentic markers.

Analysis of lesion-containing oligonucleotides. Each sample, was subjected to 3 different assays (see page 16) outlined earlier, that is: (i) formic acid hydrolysis; (ii) enzymatic hydrolysis; and (iii) alkaline hydrolysis. However, because of the large radioactively-labelled precursor pools present in the TCA-soluble fraction derived from CHO cells, a greater amount of radioactivity (5 - 10 x 10^4 dpm) was required for each assay than had been used in the case of human cells. Species present were quantified by HPLC analysis.

RESULTS

Relative sensitivity of CHO and human cells to [^{3}H]-thymidine labelling. All three CHO cell lines used in this study, including the parent line AA8, showed hypersensitivity to [^{3}H]-thymidine labelling of their DNA. Incubation in radioactive medium for periods exceeding 24 hr resulted in substantial cell loss (~90% of cells detached) regardless of the [^{3}H]-thymidine concentration (0.5 - 5 µCi/ml) used (data not shown). On the other hand, the human fibroblast strain, GM38, was routinely incubated in tritiated medium for up to 72 hr without any apparent deleterious effects. In addition, post-UV incubation of GM38 for periods exceeding 48 hr were employed without substantial cell loss. However, with the CHO cell lines, cell lysis and associated DNA breakdown coincided with repair times greater than 12 hr. Thus, the acid-soluble fractions collected from rodent cultures undergoing longer incubations had an unusually high thymidine content, making these samples difficult to analyze.

Recovery of TCA-soluble material from normal CHO and human cells. One means of assessing the repair capacity of cells is to isolate the lesion-containing excision fragments by lysing [^{3}H]-thymidine labelled cells in 5% TCA at various times after UV irradiation (La Belle and Linn, 1982). The resulting TCA-soluble fractions from normal CHO cells, presented in Table VII, are compared to data from the same experiment performed with normal human fibroblasts. Clearly, the TCA-soluble fraction collected from sham-irradiated CHO cells constituted a larger proportion of the total radioactivity (0.042%) than in the case of normal human cells (0.028%). In both cell types, a significant increase in the TCA-soluble fraction as a function of post-UV incubation time was observed (to 0.191% and 0.119%, respectively), indicating that CHO cells, like normal human cells, are capable of releasing radiolabel from high molecular weight DNA upon post-UV incubation.

Analysis of the TCA-soluble material by SAX-HPLC. The time- and UV-dependent increase in acid-soluble fraction could be the result of either nucleotide excision repair or UV-induced DNA breakdown from dead or dying cells resulting partially from the tritium labelling of the genomic DNA. To elucidate the nature of the TCA-soluble radiolabel isolated from sham-irradiated cells, SAX-HPLC was employed. The chromatographic profiles from unirradiated CHO or human fibroblasts are depicted in Figure 12, panels A and B, respectively.

In the case of CHO cells, 5.7% of total radioactivity was associated with thymidine (and thymine) and the remainder with phosphorylated derivatives of thymidine, i.e. TMP (5.3%), TDP (9.3%), and TTP (66.0%). A similar profile was obtained for human cells, although the distribution of radiolabel in the various compounds was strikingly different. The majority of the TCA-soluble material in this case was thymidine and thymine (56.7% of total radioactivity) with TMP, TDP, and TTP representing 8.0%, 3.5%, and 11.3% of the total radioactivity, respectively. Formic acid hydrolysis revealed that none of the aforementioned fractions contained cyclobutane dimers (data not shown), as expected.

Direct SAX-HPLC analysis of the TCA-soluble fraction isolated from post-UV incubated (40 J/m^2; 12-hr repair) normal CHO cells resulted in the chromatography profile shown in Figure 12C. In addition to the peaks obtained from sham-irradiated cells, a novel peak (eluting at fractions 39-42), associated with 11.7% of total radioactivity, was shown by formic acid hydrolysis to represent excised oligonucleotides containing cyclobutane dimers. Surprisingly, however, there was also a significant number of cyclobutane dimers (~20%) found within the void volume peak known to contain uncharged molecules (data not shown). A similar analysis of the TCA-soluble sample recovered from post-UV incubated (40 J/m^2; 24 hr repair) human fibroblasts (Figure 12D) revealed that 50.1% of the radiolabel eluted in a UV-dependent peak. The similarity in retention time of this novel peak in CHO and human cells suggests that those excision fragments retained by SAX chromatography, from both sources, were roughly identical in size.

It is quite obvious, especially in the case of CHO cells which are capable of little cyclobutane dimer excision, that the presence of an excessive amount of thymidine species (~80% of total radiolabel in the excision fragments from 12 hr) might interfere with accurate measurement of dimer content by formic acid hydrolysis (see below). However, attempts to eliminate the precursor pools by extensive post-labelling chase periods (up to 72 hr) were unsuccessful.

Measurement of cyclobutane dimers in excision fragments from CHO and human cells. To demonstrate that the TCA-soluble material from post-UV incubated CHO and human cells contained cyclobutane dimers, samples of acid-soluble material were hydrolysed in formic acid, and the hydrolysates were examined by reverse-phase HPLC (see Table VIII). Typical radioactivity profiles for sham-irradiated and UV-irradiated (40 J/m^2) CHO cells are compared to profiles for human fibroblasts (Figure 13). Clearly, as expected, sham-irradiated samples did not contain cyclobutane dimers. After a 12-h repair period, the TCA-soluble fraction isolated from UV-treated CHO cells had only ~6.0% of the total radioactivity associated with cyclobutane dimers (T<>U, 2.0%; T<>T, 4.0%). It is of interest to notice that a substantial amount of radioactivity (6-10%) eluted after the thymine peak (see Figure 13B). These compounds, which are essentially nonexistent in acid hydrolysates from human origin (see Figure 13D), are now known to be related to (6-4) photoproducts (Liuzzi *et al.*, submitted for publication). Results obtained for other post-UV incubation times are presented in Table VIII and can be compared to the same experiment in normal human fibroblasts (GM38) presented in Table IV of Chapter 2 (see page 44). These data clearly indicate that CHO cells, like human cells, excised cyclobutane dimers from their DNA.

Measurement of (6-4) photoproducts and modified cyclobutane dimers in excision fragments from CHO cells. Formic acid hydrolysis allows measurement only of the total number of cyclobutane dimers irrespective of configuration, and (6-4) photoproducts cannot be quantitated by this harsh treatment. To assess whether excision fragments from rodent cells contained (6-4) photoproducts and/or modified cyclobutane dimers, the enzymatic digestion procedure was utilized and the hydrolysates were examined by reverse-phase HPLC, either immediately or following hot alkaline treatment. Chromatograms resulting from enzymatic hydrolysis of samples taken from sham-irradiated and from post-UV incubated (40 J/m^2; 12 hr repair) normal CHO cells are diagrammed in Figure 14, panels A and B, respectively. In the case of sham-irradiated cells, the majority of radioactivity (92.5%) eluted with the thymidine marker. Hydrolysis of the UV-irradiated sample gave rise to five UV-dependent peaks, each identifiable as a specific type of photoproduct by its characteristic retention time. Unmodified cyclobutane dimers represented only a minority, i.e. 0.9%, of the radiolabel (d-T<p>C, 0.3%; d-T<p>T, 0.6%). Significantly more radioactivity (4.0% of the total) was contained within the peak corresponding to dT<>dT. The majority of label (16.3%), however, eluted at the retention time of the comigrating modified thymidine-cytidine dimers and (6-4) photoproducts, dT<>dC + d-T[p]Py. Subsequent alkaline hydrolysis revealed that the contribution to this peak by (6-4) photoproducts was 13.6/16.3 x 100% = 83% (see Table IX). For comparative purposes, a similar experiment for normal human fibroblasts is presented in Figure 14, panels C and D. Again, the majority of radiolabel (90.4%) from sham-treated human fibroblasts was released as thymidine by enzymatic digestion. Similar treatment of the TCA-soluble fraction from irradiated cells yielded the same 5 UV-dependent peaks corresponding to the various photolesions. A greater percentage of the radiolabel was found to elute at the retention time of dT<>dT (17.1%) than at that of d-T<p>T (4.1%), again resembling the CHO sample. The major UV-dependent peak (19.3%) also corresponded to the molecules dT<>dC + d-T[p]Py, but in sharp contrast to the CHO profile, where this major lesion-containing peak consisted almost entirely of (6-4) photoproducts, alkaline hydrolysis revealed that dT<>dC was a significant contributor to this peak [48% corresponding to this molecule and 52% representing the (6-4) photoproducts (see also Table IX)]. Results for other repair times in the normal CHO cell line are summarized in Table IX. [Note: Compare to the same experiment in human fibroblasts (Chapter 2, Table IV, page 44).] Clearly the major labelled photoproduct found in the TCA-soluble fraction of post-UV incubated CHO cells, regardless of the repair time, was the (6-4) photoproduct. The radiolabel in all photoproducts peaked at 6 hr in rodent cells, and subsequently diminished. This apparent decrease was most likely due to increased cell death and general DNA breakdown with extended repair times since the UV-dependent peak seen on the SAX profile have the same retention time regardless of repair period (data not shown). For human cells, the percentage of counts in the dT<>dC + d-T[p]Py peak was also found to be greatest after 6 hr post UV. However, the contribution to this peak by dT<>dC, and the radiolabel in each of the other cyclobutane dimer peaks, continued to increase up to 24 hr following UV-irradiation.

Disappearance of UV Photoproducts from Genomic DNA. Genomic DNA was isolated from post-UV incubated CHO cells, hydrolysed with snake venom phosphodiesterase and calf alkaline phosphatase, and subsequently dialysed overnight in order to remove mononucleosides, thereby enriching for the lesion-containing trinucleotides. This latter procedure did not decrease the amount of radioactivity retained in the dialysis bag to the same extent as it had when the same procedure had been performed with human cells (~10% retained as compared to less than 1%), but this higher contamination of mononucleosides did not interfere with subsequent analysis. However, a larger amount of radioactivity was required for evaluation in the nuclease P1 digestion/ HPLC analysis (~1 x 10^7 dpm) which followed.

As is evident from Table X, there was no significant decrease in radiolabel migrating at the retention times of the two cyclobutane dimer species, d-T<p>C and d-T<p>T, with increasing repair time. The percentage of radioactivity in d-T<p>C decreased from 0.342% to 0.306 % in 24 hr, while that associated with d-T<p>T remained unchanged at 0.82%. In contrast, the radioactivity corresponding to (6-4) photoproducts decreased to almost background level by 6 hr post UV (from 0.173% of total radiolabel to 0.045%).

Although there was a small amount of the total radioactivity migrating at the retention time of modified dimers, these data have not been included, largely because measurement of these lesions in genomic DNA is not quantitative by this method. The trinucleotide containing a modified dimer (i.e. dNpdT<>dPy) would have only one internal charge. As can be seen in Figure 2 (Chapter 1, page 48), molecules containing only one charge were retained very poorly by dialysis, whereas those containing two internal charges (such as is the case for intact lesion-containing trinucleotides) were almost completely conserved, even after 24 hr of dialysis.

Time course for (6-4) photoproduct and cyclobutane dimer repair in CHO and human cells. The absolute number of T[p]Py (6-4) photoproducts, as well as T^C and T^T cyclobutane dimers, removed from, or remaining, in the genomic DNA of rodent and human cells, in the 24 hr following irradiation with 40 J/m^2, are presented in Table XI and Figures 15 and 16. The data for human has been derived from Tables III and V (pages 43 and 45) and are presented again for comparison purposes. The assumptions used to calculate each lesion remaining or excised per rodent cell are summarized in the corresponding table legends. As is the case for human cells (see Chapter 2, page 24), 40 J/m^2 is well above the saturation dose of ~15 J/m^2 for cyclobutane dimer repair in CHO cells (Yagi *et al.*, 1984; Mitchell *et al.*, 1988). (The D_{10} value for normal CHO cells is 10-15 J/m^2 depending on the strain studied.) However, (6-4) photoproduct repair does not appear to be saturated below 40 J/m^2 in either human or CHO cells.

As is clearly evident in Figure 15, CHO and human cells showed virtually identical kinetics for the (6-4) photoproduct repair, with excision of these lesions occurring rapidly, so as to be completed within 6 hr post UV. For example, in CHO cells, the 5.5 x 10^5 T[p]Py (6-4) photoproducts initially induced was reduced to 3 x 10^4 by 6 hr post UV. In sharp contrast, the rate of cyclobutane dimer removal was much slower in both human and CHO cells. In GM38 cells, repair was constant over the 24-hr period studied, the numbers of T^C and T^T lesions decreasing from 1.21 x 10^6 to 6.8 x 10^5 and from 1.64 x 10^6 to 1.44 x 10^6, respectively. Repair of cyclobutane dimers was minimal in the rodent cells, the number of T^C lesions decreasing from 1.30 x 10^6 to 1.11 x 10^6 and T^T dimers from 1.58 x 10^6 to 1.49 x 10^6, corresponding to only 15% of what normal human cells were capable of removing in the same 24-hr period. This corresponds to repair of only 3 - 4% of the dimers induced by 40 J/m^2 in 24 hr as compared to

~25% for human cells.

Time course for unmodified versus modified cyclobutane dimers in CHO and human cells. Both post-UV incubated CHO cells and human fibroblasts were found to accumulate two types of cyclobutane dimers in the acid-soluble fraction, namely those with an intact and those with a cleaved intradimer phosphodiester linkage (see Table IX). Calculation of the % of total dimers with an intact linkage reveals that this value decreased with extended post-UV incubation times. Therefore, in both CHO and human cells, cyclobutane dimers removed at early post-UV times (e.g., 3 hr) had a relatively high probability of being unmodified (~40%) whereas those repaired later (e.g., 24 hr) were more likely to be modified (~20% unmodified).

Repair of UV lesions in two UV sensitive CHO mutants. The two mutant cell lines, UV-4 and UV-5, belong to different complementation groups and, therefore, the basic genetic defect which results in UV sensitivity, is presumably different in the two cell lines. Both mutants were studied for their ability to repair UV photoproducts. Neither was found to accumulate either cyclobutane dimers or d-T[p]Py (6-4) photoproducts in the TCA-soluble material derived from post-UV incubated cultures (data not shown). This indicates that both mutants have lost their ability to repair (6-4) photoproducts as well as the small amount of cyclobutane dimer repair carried out by the parent strain, AA8.

DISCUSSION

Various means of repairing UV-induced DNA damage exist in nature, including photoreactivating enzymes (Sutherland, 1978), dimer-specific DNA glycosylases (Haseltine *et al.*, 1980; Radany and Friedberg, 1980), and endonuclease complexes (Yeung *et al.*, 1983; Sancar and Rupp, 1983). In human cells, a UV-specific glycosylase activity is not detectable (La Belle and Linn, 1982), and although repair is believed to involve a mechanism analogous to UvrABC of *E. coli*, genetics indicate that these systems differ (Hanawalt *et al.*, 1979). Consequently, it is not a safe assumption that any two species repair UV-induced DNA damage using equivalent pathways. With this in mind, it was of great interest to assess CHO cells for the presence or absence of a repair-related phenomenon which had been previously described for human fibroblasts. In particular, earlier work had shown that excision fragments isolated from post-UV incubated normal human fibroblasts contained cyclobutane dimers with a cleaved intradimer phosphodiester bond (Paterson *et al.*, 1984; Weinfeld *et al.*, 1986), and it had been proposed that this may be the initial reaction in the multistep pathway responsible for the removal of cyclobutane dimers from the DNA (Paterson *et al.*, 1987). In order to ascertain whether or not this is also a characteristic of UV repair in CHO cells, lesions contained within excision fragments isolated from post-UV incubated CHO cells, as well as the lesions remaining in the genomic DNA, were examined. This study demonstrates that human and CHO cells have common repair characteristics, with only one notable exception, namely less efficient cyclobutane dimer repair.

It is well-documented that rodent cells are deficient relative to human cells in cyclobutane dimer removal (Meyn *et al.*, 1974; Yagi, 1982; Vijg, *et al.*, 1984; Bohr *et al.*, 1985). In fact, it has been claimed that these lesions are undetectable in the TCA-soluble fraction from post-UV incubated cells (Klímek, 1966; Trosko and Kasschau, 1967). In this study, a significantly higher percentage of total radiolabel was recovered in the TCA-soluble material from sham-irradiated CHO cells than nonirradiated human cells. The abundance of this acid-soluble radioactivity could not be reduced even with extended post-labelling chase periods. SAX chromatography clearly showed that whereas thymidine was the predominant labelled species in material isolated from sham-irradiated human fibroblasts, the majority of acid-soluble radiolabel in CHO cells (whether UV-irradiated or not) was phosphorylated derivatives of thymidine (particularly TTP), and therefore could not have resulted from DNA breakdown. Why these cells contain large nucleotide precursor pools is unknown, but it has been previously documented (Vagi *et al.*, 1984). This background noise could explain the difficulty others have had detecting cyclobutane dimers in TCA-soluble material isolated from UV-irradiated CHO cells. CHO cells do, however, have a much reduced ability to repair these lesions, with only ~15% of the repair capacity displayed by human cells (see Table XI). In fact, so few of these lesions are repaired that it was impossible to detect it by monitoring genomic DNA alone. This is because of the difficulty in distinguishing a small reduction in the number of lesions present from experimental error.

It is intriguing that, for both CHO and human cells, cyclobutane dimers were detected in the void volume from the SAX column. Such molecules are uncharged, thereby implying the presence of cyclobutane dimer-containing dinucleosides. It is unknown at this stage whether dimers can be removed from the genome in this molecular configuration or whether they resulted from nuclease degradation of the excised oligonucleotides. Most (80%) of the cyclobutane dimers were, however, retained by the SAX column. Although these excision fragments eluted in a broad peak (characteristic of a heterogeneous population of molecules), the chromatographic behavior was the same for both CHO and human cells. Molecules migrating in this position correspond to oligonucleotides having approximately 4 to 6 internal phosphate groups. Although *in vitro* nucleotide excision studies have shown that UV lesions are excised from double-stranded DNA in a fragment of approximately 29 nucleotides in length (Huang *et al.*, 1992), the above result implies that in both rodent and human cells lesions are either excised, *in vivo*, within fragments of 5 - 7 nucleotides (see Chapter 2, page 38) or, alternatively, the excision fragments are processed in the same manner following their removal from the genomic DNA.

Of the cyclobutane dimers which are removed from genomic DNA in both CHO and human cells, the majority were found in excision fragments with a cleaved intradimer phosphodiester bond. The proportion of modified dimers in the TCA-soluble fraction increased with repair time and thus it might be argued that modification represents a post-excision event rather than a pre-excision event. Prior observations by us and other groups, however, argue against this. First, we have previously shown that photoenzymatic reversal of the cyclobutane dimers remaining in the genome of post-UV incubated XP group D cells (Paterson *et al.*, 1984), resulted in the accumulation of single-stranded breaks. This implies that intradimer phosphodiester bonds had been ruptured while these lesions were still in the genome. It was postulated at the time that intradimer cleavage was the initial pre-incision event in the multistep nucleotide excision repair pathway operating on these lesions and that XP group D cells aborted repair at this stage. Second, Mitchell and coworkers (1988) have shown that as post-UV repair progressed in CHO cells, the binding of antibodies specific to cyclobutane dimers decreased faster than the number of sites sensitive to the incising activity of T4 UV endonuclease. It was postulated that dimer modification would precede excision and that once modified, cyclobutane dimers could not be recognized by the antibody. To reconcile their findings with data regarding DNA replication on a UV-damaged template (Konze-Thomas *et al.*, 1982; Dahle *et al.*, 1980), Mitchell and coworkers (1988) suggested that cyclobutane dimers were modified in order to allow replication to proceed beyond these lesions. Third, Pirsel *et al.* (1989) demonstrated the transient appearance of photolyase-induced breaks in post-UV incubated hamster fetal cells. Finally, work in our lab has shown the preferential accumulation of modified dimers in transcriptionally active genes of CHO cells and their transient appearance in the same genomic regions of human cells (see Chapter 8). These collective results, therefore, imply that the modification of cyclobutane dimers is a pre-excision event and does not occur solely from post-excision processing of excision fragments, although limited post-excision modification cannot be excluded.

It is significant that not all cyclobutane dimers found in excision fragments were modified (in either CHO or human cells). Rather, some retained an intact intradimer phosphodiester bond. A higher proportion of these lesions were unmodified at early repair times than at later times. If modification is related to polymerase and/or transcription bypass, it is possible that they represent cyclobutane dimers which had been removed from DNA not yet prepared for replication and/or transcription, and have therefore not been subjected to the modification. Since DNA replication and transcription are inhibited for a period of time immediately following UV-irradiation, a larger percentage of the cyclobutane dimers excised at early times would be expected to be unmodified.

Given the high UV survival displayed by CHO cells in spite of their relatively poor ability to excise cyclobutane dimers, it is obvious that they must have some means of circumventing this unrepaired UV-damage. If these lesions are in fact preferentially removed from transcriptionally active genes as has been proposed (Bohr *et al.*, 1985), it is possible that their persistence in the remainder of the genome is tolerated by cleaving the intradimer backbone, thereby ensuring that replication is not interrupted. Although persisting DNA damage blocks replication (Hanawalt *et al.*, 1979), replicated and unreplicated DNA are found to have the same dimer content in post-UV incubated hamster cells (Meyn *et al.*, 1974). Although recombination repair would bring this about, the implication is that, *in vivo*, cyclobutane dimers do not completely block DNA synthesis, but are somehow tolerated. A reasonable hypothesis is that tolerance may be achieved by dimer modification as a preamble to replication. In such a case, unless cleavage of the intradimer phosphodiester bond converts this otherwise non-coding lesion into a molecular configuration capable of base-pairing during replication, rodent cells would be expected to be more susceptible to UV-induced mutagenesis. This not being the situation (Zelle *et al.*, 1980), if modification permits replication bypass, it would need to promote base-pairing. Preliminary *in vitro* experiments (see Chapter 8), using *E. coli* DNA polymerase I, indicate that at least this replicating enzyme is not able to bypass a modified dimer. However, until a major replication enzyme, such as *E. coli* Polymerase III or a mammalian polymerase, or an *in vivo* system can be tested, this is hardly a conclusive observation.

Another possible function for intradimer phosphodiester cleavage is in allowing the transcription apparatus to bypass this lesion. As indicated above, there is evidence from our laboratory that what has actually been described as preferential repair of transcriptionally active genes is, in fact, dimer modification. (The relevant experiments are discussed in more detail in Chapter 8.) Preliminary *in vitro* transcription assays on a substrate containing a modified dimer also support this premise (see Chapter 8). In the same way that preferential repair has been proposed to promote high cell survival of rodent cells in the absence of efficient overall dimer removal (Bohr *et al.*, 1985; Bohr *et al.*, 1987), so would modification if it allowed the transcription machinery to bypass this otherwise polymerase-blocking lesion (Selby and Sancar, 1990). However, it must be remembered that this ability to modify cyclobutane dimers is not unique to rodent cells. In repair proficient human cells, the majority of excised cyclobutane dimers had also been modified. This may indicate that for whatever reason cyclobutane dimers are modified, evolutionarily it is a ubiquitous [the existence of intradimer phosphodiesterase cleavage in *E. coli* supports this notion (see Chapter 7)] and essential phenomenon. The poor overall cyclobutane dimer repair found in rodent cells may merely indicate the loss of the superfluous function of cyclobutane dimer removal, or the existence of a hyperefficient bypass mechanism compared to human cells.

In sharp contrast to the general inability of CHO cells to remove cyclobutane dimers, these cells displayed a capacity equivalent to that of human cells, to excise (6-4) photoproducts. According to excision fragment data, both cell types excised ~4 x 10^5 T[p]Py (6-4) photoproducts per cell in approximately 6 hr. As indicated above, cyclobutane dimer removal was much slower and less extensive. This difference in the relative percentages and kinetics with which these two classes of lesion were repaired supports previous data based on the disappearance of specific antibody-binding sites (Mitchell *et al.*, 1985; Mitchell *et al.*, 1988).

Although the (6-4) photoproducts removed from genomic DNA could be accounted for (within experimental error) by their appearance in excision fragments for human cells, the data are not as convincing for CHO cells. There appeared to be almost twice as many (6-4) photoproducts induced in the genomic DNA as were repaired, even though the excision fragment data suggest that all of these lesions were removed (i.e there was no further accumulation of these lesions in the TCA-soluble material after ~6 hr post UV). It is improbable that this represents a discrete region of the genome that is repaired since radioimmunoassay studies (Mitchell *et al.*, 1985) demonstrate that these lesions are removed in their entirety by ~6 hr. It is more likely that this represents a limitation in the accuracy of the method for estimating the number of lesions remaining in high-molecular-weight DNA.

The prominence of (6-4) photoproduct repair reinforces the current belief that, whereas cyclobutane dimers are the major mutagenic lesion induced by UV light (Glickman *et al.*, 1986; Protić-Sabljić *et al.*, 1986), (6-4) photoproducts represent the major cytotoxic damage (Konze-Thomas *et al.*, 1982; Mitchell *et al.*, 1988; Cleaver *et al.*, 1988), although (6-4) photoproducts can still be shown to be mutagenic (Franklin and Haseltine, 1986). The importance of (6-4) photoproduct repair to cell survival has been suggested several times, either directly or indirectly, in recent literature. First of all, blocks to replication caused by UV irradiation were found to occur with a frequency lower than the expected cyclobutane dimer frequency (Dahle *et al.*, 1980). Secondly, Mitchell (1988) concluded that (6-4) photoproduct repair was responsible and necessary for split-dose recovery in UV-irradiated CHO cells. Finally, the transfection of the CHO UV-sensitive mutant, UV-5, with the phage T4 *denV* gene, resulted in only intermediate UV survival (Valerie *et al.*, 1985). In our assay, UV-5 could not excise either cyclobutane dimers or (6-4) photoproducts. The *denV* gene product is a cyclobutane dimer-specific endonuclease and therefore the denV-transformed cell line would only be able to repair cyclobutane dimers from its DNA. That this new ability of the transformed cell line brings about only intermediate cell survival implies that the repair of the (6-4) photoproducts represents a critical cellular event. The partial recovery of UV-sensitivity does, however, indicate that cyclobutane dimers, too, have a role in cell killing.

Rapid (6-4) photoproduct repair may help to explain why, whereas the majority of cyclobutane dimers recovered in excised oligonucleotides had a cleaved internal phosphodiester linkage, (6-4) photoproducts always contained an intact intradimer phosphodiester backbone (see Figure 9, page 55). If cyclobutane dimers are, in fact, modified to allow replication and/or transcription past this damage, (6-4) photoproducts are either (i) removed prior to recovery from inhibition of DNA and RNA synthesis which follows UV-irradiation and therefore need not be modified or (ii) incapable of being modified [or, alternatively, intra(6-4)lesion phosphodiester cleavage may not alleviate the block to replication and/or transcription] and, therefore, since they threaten to block polymerase progression, a rapid (6-4) photoproduct repair system has evolved.

As discussed in Chapter 2, several observations lead to the conclusion that there exists more than one repair mechanism operating on UV-induced damage in mammalian DNA. To reiterate and add further to this, firstly, cyclobutane dimers and (6-4) photoproducts are removed with very different kinetics (Mitchell *et al.*, 1985; this study, Figures 15 and 16). Secondly, CHO cells have a marked difference in their ability to repair the two classes of lesions (Mitchell *et al.*, 1985; this study). Thirdly, cyclobutane dimers are often found in excision fragments with a cleaved intradimer phosphodiester bond (Weinfeld *et al.*, 1986; this study), whereas this bond in (6-4) photoproducts appears to be always intact. Of course, the first two points could be attributed to a higher affinity of a limiting recognition/incision complex for (6-4) photoproducts (Jones *et al.*, 1992), but results presented in Chapter 4 argue against this. In addition, studies on lesion repair in mouse skin question this notion. These results indicate that data acquired from tissue culture do not always reflect the true *in vivo* situation. Rodent cells, *in situ*, possess an early rapid mode of cyclobutane dimer repair which is lost upon cell culturing, although (6-4) photoproduct removal remains unaltered (Mullaart *et al.*, 1988). Therefore, some difference in the mode of repair of these two types of lesions must exist. Since all photoproducts appear to be removed by nucleotide excision repair, the deduction to be drawn from these collective findings is that the initial recognition and/or incision steps must be different for (6-4) photoproducts and cyclobutane dimers, and possibly even for modified versus intact dimers.

strain	UV fluence (J/m^2)	repair time (h)	[^{3}H]Thy (dpm/dish)		% Thy in acid soluble [A/B]= [C]
			acid soluble [A]	total x10^{-6} [B]	
AA8	0	12	71,390	170	0.042
AA8	40	0	72,670	174	0.042
AA8	40	3	71,590	168	0.043
AA8	40	6	78,680	134	0.059
AA8	40	12	97,830	113	0.087
AA8	40	24	125,880	66.0	0.191
GM38	0	24	8900	32.1	0.028
GM38	40	0	9700	32.9	0.029
GM38	40	3	13,500	32.8	0.041
GM38	40	6	17,950	31.5	0.057
GM38	40	12	30,950	33.3	0.093
GM38	40	24	39,420	33.0	0.119

TABLE VII Radioactivity recovered in TCA-insoluble and -soluble fractions from CHO cells (AA8) or human fibroblasts (GM38) after no treatment or 40 J/m^2 and various repair periods.

UV fluence (J/m^2)	repair time (h)	%[^{3}H]Thy in major peaks			
		T<>U	T<>T	Thy	Σ
0	12	0.0	0.0	90.3	90.3
40	0	0.0	0.0	91.2	91.2
40	3	0.9	4.1	79.3	84.3
40	6	2.9	6.1	74.1	83.1
40	12	2.0	4.0	75.5	81.5
40	24	1.0	2.5	81.4	84.9

TABLE VIII Percentage of total radiolabel in major peaks after formic acid hydrolysis of TCA-soluble fractions isolated from post-UV incubated AA8 cells. Note: The percentages of total counts do not add up to 100% because a minor fraction of counts is distributed over the remainder of the chromatogram.

UV fluence (J/m^2)	repair time (hr)	%[^{3}H]Thy in each major peak										
		enzymatic hydrolysis					alkaline hydrolysis					
		d-T<p>C	d-T<p>T	dT<>dC d-T[p]Py	dT<>dT	dT	T[]Py	d-T<p>C	d-T<p>T	dT<>dC	dT<>dT	dT
0	12	0.0	0.0	0.0	0.0	92.5	0.0	0.0	0.0	0.0	0.0	92.1
40	0	0.0	0.0	0.0	0.0	93.4	0.0	0.0	0.0	0.0	0.0	92.8
40	3	1.4	1.8	18.8	4.7	51.3	19.5	2.2	1.9	2.3	2.4	52.6
40	6	1.7	2.0	23.4	9.6	53.2	27.1	1.8	1.0	3.2	5.3	51.1
40	12	0.3	0.6	16.3	4.0	68.2	21.2	0.6	0.5	2.7	3.8	69.1
40	24	0.6	0.4	6.9	2.4	87.3	7.8	0.8	0.4	0.8	2.6	84.8

TABLE IX Percentage of total radiolabel in major peaks after enzymatic or alkaline hydrolysis of TCA-soluble material from post-UV incubated AA8. Note: The percentages of total counts do not add up to 100% because a minor fraction of counts is widely distributed over the remainder of the chromatogram.

repair time (hr)	dpm before dialysis	dpm after dialysis	% dpm retained	% dpm in d-T<p>C	% dpm in d-T<p>T	% dpm in d-T[p]Py
0	17,500,500	2,098,300	11.99	0.342	0.82	0.173
1	20,068,900	2,039,000	10.16	0.388	0.95	0.191
2	16,468,200	1,571,100	9.54	0.402	1.01	0.154
3	18,493,400	1,732,800	9.37	0.406	1.04	0.114
4	21,602,200	2,095,400	10.22	0.368	0.92	0.084
6	21,594,000	2,198,300	10.33	0.362	0.94	0.045
12	20,500,200	2,308,300	11.26	0.345	0.87	0.033
24	13,355,000	1,458,400	11.35	0.306	0.82	0.031
sham-irradiated	18,485,100	1,948,300	10.54	0.0	0.0	0.034

TABLE X Radioactivity retained after dialysis of enzymatically hydrolysed UV-irradiated (40 J/m^2) AA8 genomic DNA and contained in the various photolesion peaks after subsequent nuclease P1 digestion.

repair time (hr)	T^C dimers				T^T dimers				(6-4) photoproducts			
	remaining in genomic DNA (x10^{-4})		recovered from excision fragments (x10^{-4})		remaining in genomic DNA (x10^{-4})		recovered from excision fragments (x10^{-4})		remaining in genomic DNA (x10^{-4})		recovered from excision fragments (x10^{-4})	
	AA8	GM38	AA8	GM38	AA8	GM38	AA8	GM38	AA8	GM38	AA8	GM38
0	130	121	0	0	158	164	0	0	55	43	0	0
1	126	nd	nd	nd	154	nd	nd	nd	50	nd	nd	nd
2	122	nd	nd	nd	154	nd	nd	nd	35	nd	nd	nd
3	122	119	1	2	155	164	3	2	22	24	23	13
4	120	nd	nd	nd	150	nd	nd	nd	16	nd	nd	nd
6	118	116	5	7	154	164	6	4	3	7	38	42
12	124	103	6	23	156	157	6	14	1	1	38	42
24	111	68	6	52	149	144	8	44	0	0	37	39

TABLE XI UV photoproducts remaining in genomic DNA or recovered in excision fragments for post-UV (40 J/m^2) incubated normal CHO (AA8) and human (GM38) cells. Data for GM38 are taken from Tables III and V in Chapter 2 (see pages 43 and 45). Calculations for AA8 are based on assumptions presented in the legends of these same tables. nd = not done

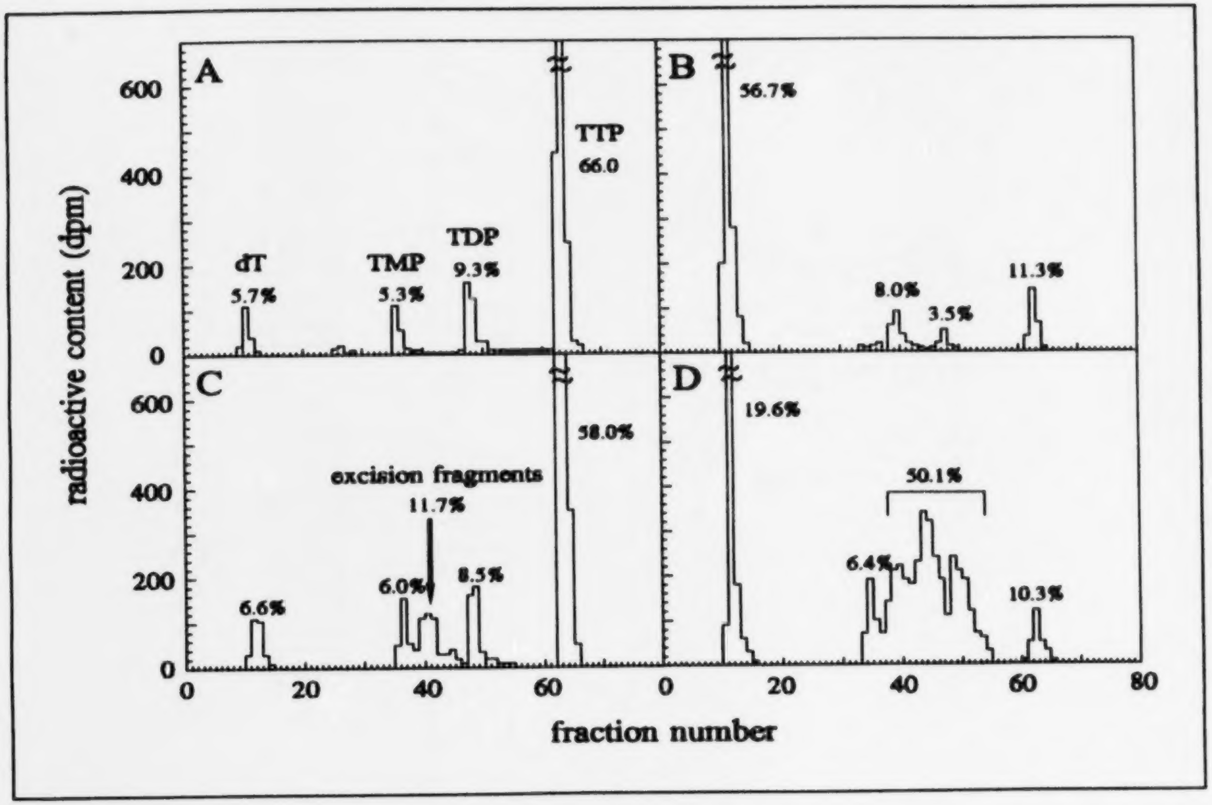


FIGURE 12 SAX-HPLC radioactivity profiles of TCA-soluble material from CHO (AA8) and human (GM38) cells. **A.** Sham-irradiated AA8 cells. **B.** Sham-irradiated GM38. **C.** Post-UV (40 J/m^2) incubated (12 hr) AA8 cells. **D.** Post-UV (40 J/m^2) incubated (24 hr) GM38. Numbers indicated on chromatograms refer to % of total radioactivity in each major peak.

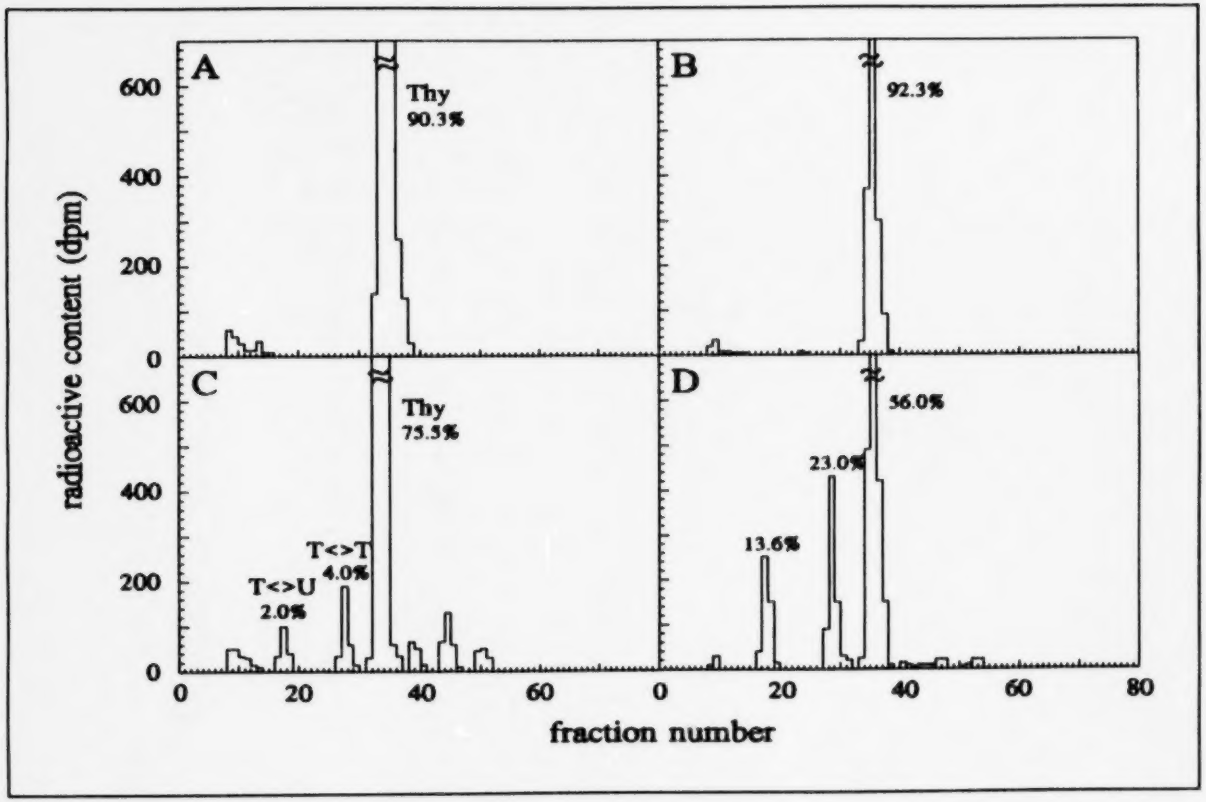


FIGURE 13 Reverse-phase HPLC radioactivity profiles of TCA-soluble material from CHO (AA8) and human (GM38) cells after formic acid hydrolysis. **A.** Sham-irradiated AA8 cells. **B.** Sham-irradiated GM38. **C.** Post-UV (40 J/m^2) incubated (12 hr) AA8 cells. **D.** Post-UV (40 J/m^2) incubated (24 hr) GM38. Numbers indicated on chromatograms refer to % of total radioactivity in each major peak.

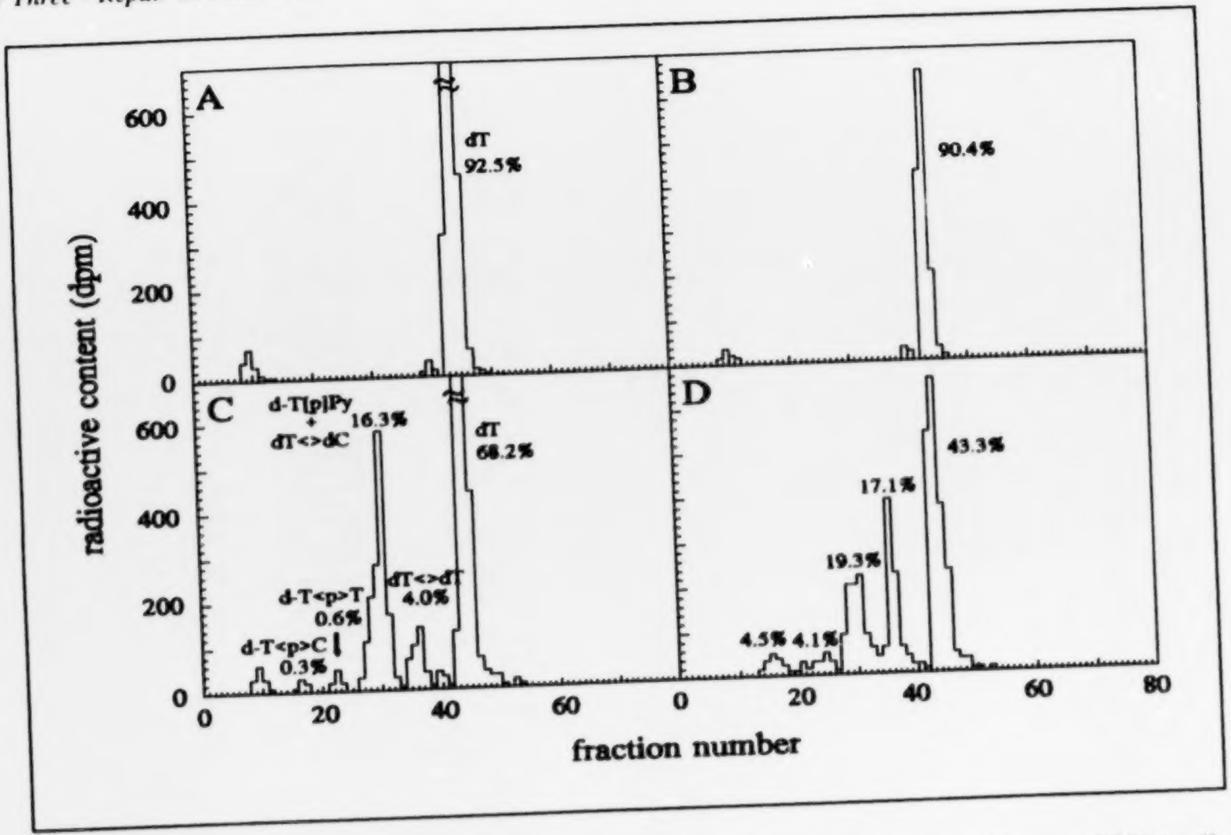


FIGURE 14 Reverse-phase HPLC radioactivity profiles of TCA-soluble material from CHO (AA8) and human (GM38) cells after enzymatic hydrolysis. **A.** Sham-irradiated AA8. **B.** Sham-irradiated GM38. **C.** Post-UV (40 J/m^2) incubated (12 hr) AA8. **D.** Post-UV (40 J/m^2) incubated (24 hr) GM38. Numbers indicated on chromatograms refer to % of total radioactivity in each major peak.

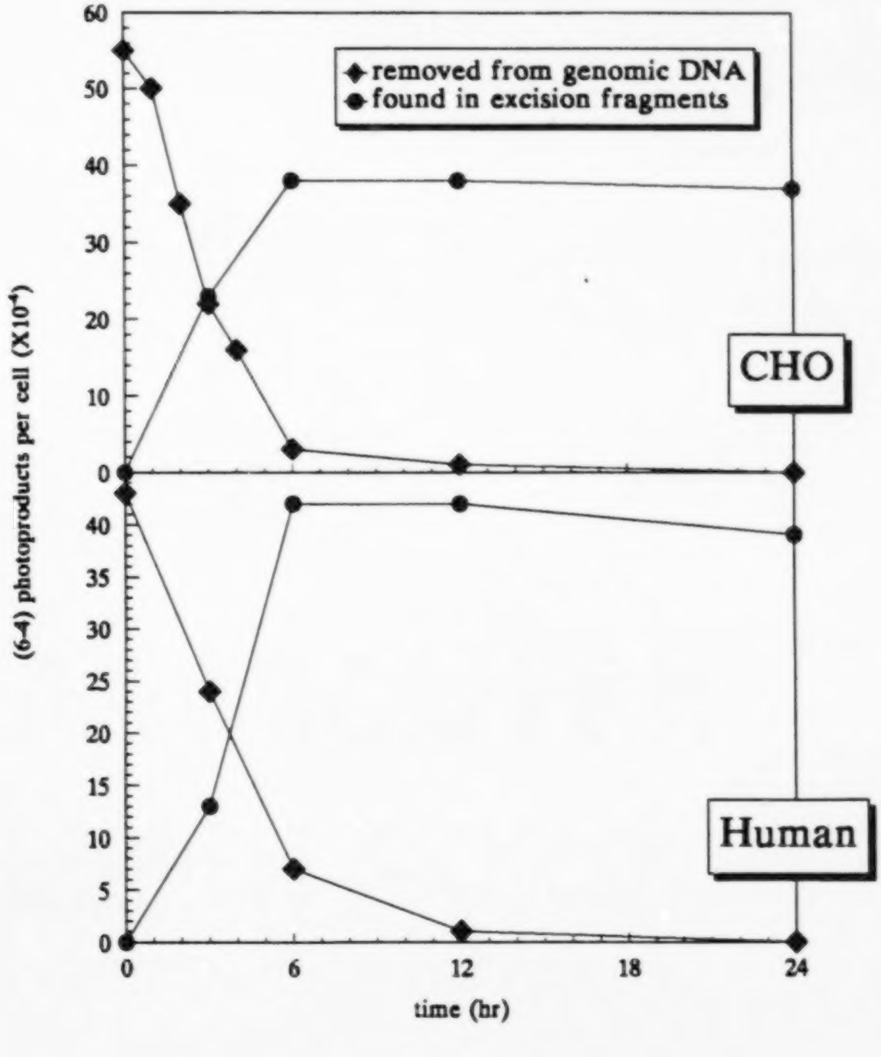


FIGURE 15 Repair of (6-4) photoproducts in CHO cells (AA8) and human fibroblasts (GM38) following 40 J/m^2 of 254-nm light. Circles indicate the accumulation of these lesions in excision fragments. Diamonds show their removal from genomic DNA.

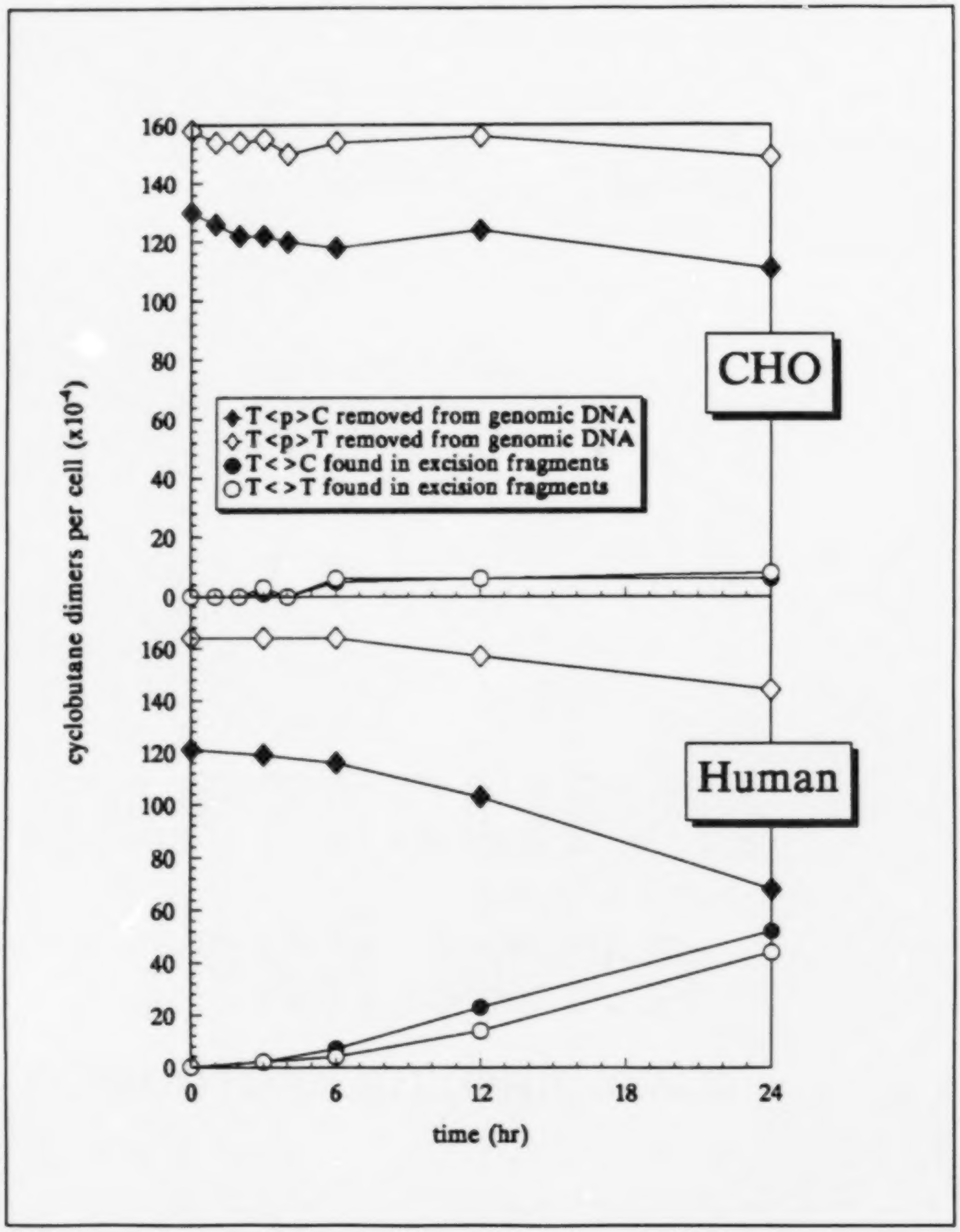


FIGURE 16 Repair of cyclobutane dimers in CHO cells (AA8) and human fibroblasts (GM38) following 40 J/m^2 of 254-nm light. Symbols depicting the various lesions are indicated in the figure legend.

CHAPTER FOUR - XERODERMA PIGMENTOSUM CELLS HAVE DIFFERENT ABILITIES TO REPAIR PYRIMIDINE-PYRIMIDONE (6-4) PHOTOPRODUCTS VERSUS CYCLOBUTANE PYRIMIDINE DIMERS

INTRODUCTION

The human genetic disorder xeroderma pigmentosum (XP) is inherited in an autosomal recessive manner. Patients with this disease have extreme sensitivity to the UV component of sunlight and display an enhanced tendency to develop skin cancer (Kraemer, 1983). Cultured fibroblasts from XP patients exhibit reduced survival following UV exposure (Andrews *et al.*, 1978; Arlett *et al.*, 1980). Using cell fusion techniques, XP cells have been divided into 8 complementation groups. One group, the so-called XP variants, is defective in postreplication repair (Lehmann *et al.*, 1975). The other 7 groups (designated complementation groups A-G) are believed to be all defective in the initial incision step of the multi-step excision repair process responsible for removing UV-induced photoproducts from the DNA (Zelle and Lohman, 1979; Kraemer, 1983; Wood *et al.*, 1988).

As mentioned previously, one commonly employed technique, used to assess the repair proficiency of an XP strain, utilizes the sensitivity of cyclobutane dimers to the strand-incising activity of the *Micrococcus luteus* UV endonuclease and measures the time-dependent disappearance of single-strand breaks from cellular DNA (Paterson *et al.*, 1981). A second independent technique measures unscheduled DNA synthesis (UDS), an indication of the cell's ability to perform DNA-repair synthesis (Cleaver, 1968). These techniques give similar results for most XP complementation groups. For example, XP-A cells have very little, if any, ability to remove cyclobutane dimers as measured by the removal of UV endonuclease-sensitive sites, and are totally deficient in DNA repair synthesis as measured by UDS. Other complementation groups (notably B, C, E, G) are only partially defective in the removal of UV endonuclease-sensitive sites and retain about the same percentage of normal UDS. XP groups D and F are, however, notable exceptions. While able to carry out 20 to 55% of normal UDS, XP-D cells are almost totally defective in the removal of UV endonuclease-sensitive sites (Paterson *et al.*, 1973; Zelle and Lohman, 1979; Kraemer, 1983). This residual level of UDS is not decreased after microinjection of purified photolyase isolated from yeast (Zwetsloot *et al.*, 1986) or *Escherichia coli*. Since *E. coli* (Brash *et al.*, 1985) and yeast (Patrick, 1970) DNA photolyases do not act on

(6-4) photoproducts, the hypothesis was entertained that the repair of part, or all, of these lesions would be responsible for the residual level of UDS.

Conversely, XP-F cells exhibit 10 to 20% of normal UDS, yet repair ~70% of the UV-sensitive sites (Zelle and Lohman, 1979; Kraemer, 1983). Studies have shown that microinjection of purified *M. luteus* UV endonuclease only partially restores UDS (~50% of normal) (de Jonge *et al.*, 1985). Furthermore, examination of the kinetics of UDS in these cells demonstrated that although they are lacking the initial rapid repair component exhibited by normal cells, the subsequent slow repair response is normal (Zelle *et al.*, 1980; Fujiwara *et al.*, 1985). After confirming the rapid repair of (6-4) photoproducts following UV irradiation (see Chapter 2), it seemed apparent that XP-F cells must be defective in the removal of this class of UV lesions.

Using the novel enzymatic hydrolysis/HPLC approach to measuring cyclobutane dimers and (6-4) photoproducts in excision fragments isolated from post-UV incubated human cells (described in detail in Chapter 2 and see Figure 1, page 47), the repair capabilities of the eight XP complementation groups, A through G and variant, have been reassessed. This has enabled confirmation of the reported repair deficiencies/proficiencies for XP groups, A, B, C, G, and variant. However, XP-D cells, while unable to repair cyclobutane dimers, retain about 40% of normal proficiency at removing (6-4) photoproducts. These latter lesions appear in excision fragments at a rate of ~40% that of normal cells in the first 6 hr following UV-irradiation, with no further accumulation after this time. XP-E cells, in contrast, repair both photolesions, but (6-4) photoproducts are removed at a markedly reduced rate. As predicted, XP group F is proficient at cyclobutane dimers repair, but totally lacking in (6-4) photoproduct removal. Together, these results indicate that in normal cells, the repair of (6-4) photoproducts is the major contributor to UDS at early repair times (~2 h), in agreement with other recent reports (Mitchell and Nairn, 1989; Jones *et al.*, 1992). The partial repair of (6-4) photoproducts in XP-D cells is sufficient to account for the previously unexplained UDS. In XP-E cells, the reduced rate at which these lesions are removed explains subnormal UDS, not a deficiency in cyclobutane dimer repair as has been reported (Zelle and Lohman, 1979; Kraemer, 1983). In XP-F cells, the total lack of repair of (6-4) photoproducts resolves the apparent conflict of abnormally low UDS. It will, therefore, be important to re-evaluate experiments which have used UDS as indicator of repair proficiency (e.g. complementation testing).

EXPERIMENTAL PROCEDURES

For more detailed experimental procedures, see Appendix A, page 254.

Cell and culture conditions. Experiments were conducted on the following eight diploid fibroblast strains established from skin biopsies of human subjects: CRL1223 (XP group A), CRL1199 (XP group B), CRL1166 (XP group C), CRL1157 (XP group D), CRL1259 (XP group E), GM3542 (XP group F), GM3021 (XP group G) and CRL1162 (XP variant). GM3542 and GM3021 were purchased from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ) whereas CRL1223, CRL1199, CRL1166, CRL1157, CRL1259, and CRL 1162 were procured from the American Type Culture Collection (Rockville, MD). All culture conditions were identical to those presented in Chapter 2 (page 14).

Other material and methods. All materials and procedures for (i) [^{3}H]thymidine-labelling of cells, (ii) UV irradiation, (iii) collection of excision fragments, (iv) formic acid hydrolysis, (v) enzymatic hydrolysis, (vi) alkaline hydrolysis, and (vii) reverse-phase HPLC have been described in Chapter 2 (pages 14-18, 273).

RESULTS

Measurement of UV lesions in excision fragments from XP fibroblasts. The TCA-soluble material isolated from post-UV incubated XP cells was analyzed in a similar fashion as had been previously done for normal human cells (Chapter 2). This procedure allowed quantitation of the various intact or modified UV lesions in all cases. The numbers of cyclobutane dimers and (6-4) photoproducts excised per cell have been calculated in a manner similar to that done for normal cells, and are based upon the same assumptions [see legends to Tables III (page 43) and V (page 45)]. The results for the various XP complementation groups are summarized in Figures 17 (cyclobutane dimer repair) and 18 [(6-4) photoproduct removal]. As well, results for the normal human cell strain (GM38) have been taken from Table V and included for comparison purposes.

UV photoproducts failed to accumulate in the TCA-soluble fraction isolated from post-UV incubated XP groups A and G cells, consistent with the failure of other assays to find repair capabilities in either cell type (Zelle and Lohman, 1979; Mitchell, 1988a). XP group C cells excised ~1.3×10^5 cyclobutane dimers and 1.9×10^5 (6-4) photoproducts in a 24-hr period following 40 J/m^2, corresponding to ~ 12% and 35% of normal proficiency, respectively. These values are in close agreement with estimates reported previously (Zelle and Lohman, 1979; Mitchell, 1988a), and may reflect domain specific repair (Mansbridge and Hanawalt, 1983; Mullenders *et al.*, 1984; Mullenders *et al.*, 1988; van Zeeland *et al.*, 1988; Kantor *et al.*, 1990). XP group B cells were even less repair competent than XP group C cells, excising less than 2×10^4 cyclobutane dimers (< 1 % normal) and 6.4×10^4 (6-4) photoproducts (~13% proficiency) in the same 24-hr post-UV period. This correlates well with the low levels of both UDS and UV-endonuclease site removal documented for XP group B. XP group D cells did not excise significant amounts of cyclobutane dimers. Only ~0.3×10^5 cyclobutane dimers were excised in a 24-hr repair period, corresponding to ~3% of the repair capacity of normal cells, as typically reported (Paterson *et al.*, 1973; Zelle and Lohman, 1979). However, during the first 6 hr of repair, XP group D cells excised ~1.9×10^5 (6-4) photoproducts, equivalent to ~40% of the repair ability of normal human cells. It is noteworthy that the level of repair of this latter class of UV lesions did not progress past that achieved by 6 hr post UV.

While XP group E cells exhibited normal removal of cyclobutane dimer, their ability to act on (6-4) photoproducts was defective, proceeding at a markedly reduced rate. By 6 hr these cells had accomplished only about 35% of normal (6-4) photoproduct excision, but by 24 hr were indistinguishable from normal cells. This is a notable contradiction to other studies which have reported defective cyclobutane dimer repair (Zelle *et al.*, 1980) and normal kinetics for (6-4) photoproduct removal (Mitchell, 1988a) using the same cell strain (CRL1259; née XP2R0). However, in support of the present results, microinjection of photoreactivating enzyme into XP-E cells has indicated slow (6-4) photoproduct repair in this same strain (Roza *et al.*, 1990).

XP group F showed a total lack of (6-4) photoproduct repair. However, using this assay system, these cells were completely normal for cyclobutane dimer removal. This, too, contradicts other studies which showed only ~ 70% of normal UV endonuclease site removal (Zelle *et al.*, 1980). Consistent with previous failures to find a repair defect in XP variant cells (Zelle and Lohman, 1979; Mitchell and Nairn, 1987), accumulation of both cyclobutane dimers and (6-4) photoproducts in variants is nearly identical to normal cells.

Modification of cyclobutane dimers found in excision fragments from XP cells. No XP strain studied had any defect in its ability to modify cyclobutane dimers. Since at least one member of each complementation group had been analyzed, this implies that intradimer phosphodiester cleavage is proficient in all XP cells. Excision fragments from those XP strains capable of excising substantial numbers of cyclobutane dimers contained the same distribution of intact and modified cyclobutane dimers as did normal cells (data not shown). At least two of those complementation groups defective in cyclobutane dimer repair (A and D) have previously been shown to accumulate these sites in their genomic DNA following UV irradiation (Paterson *et al.*, 1987).

DISCUSSION

The utility of the enzymatic hydrolysis / HPLC methodology for quantitating the repair of both cyclobutane dimers and (6-4) photoproducts by examining the TCA-soluble material isolated from post-UV incubated cells has been substantiated in Chapter 2. This technique provides excellent agreement between lesion removal as assessed by the loss of photoproducts from the genomic DNA, and as quantitated by their appearance in excision fragments. Because it allows very accurate enumeration of (6-4) photoproducts, and because it permits distinction between intact and modified cyclobutane dimers, this novel assay system has been used to re-evaluate the repair capacity of the various XP complementation groups.

The data summarized in Figures 17 and 18 reveal that each XP complementation group has a characteristic ability / inability to repair (6-4) photoproducts and cyclobutane dimers. The complete repair deficiency of XP group A cells, the subnormal ability of XP group C cells to remove both classes of UV-induced lesions, and the normal repair capacity of XP variant cells are consistent with measurements which have utilized alternate techniques (Zelle and Lohman, 1979; Mitchell, 1988a). The residual repair observed in XP-C cells may correlate with the previously described domain-specific repair (Mansbridge and Hanawalt, 1983; Mullenders *et al.*, 1984; Mullenders *et al.*, 1988; van Zeeland *et al.*, 1988) and represent limited amounts of normal repair enzymes (Karentz and Cleaver, 1986; Cleaver, 1986; Cleaver, 1987; Kantor and Elking, 1988; Kantor *et al.*, 1990). XP group G cells, like group A, are totally defective in the removal of both classes of lesions, correlating well with previous UDS and UV endonuclease site removal studies (Kraemer, 1983). Group B appears to have a very limited capacity to repair both types of lesions, displaying only about 30% of the residual competency of XP group C.

The repair phenotypes of both XP groups A and B are easily reconcilable with the proposed function of the recently cloned cognate genes. The XP-A complementing factor is believed to be a component of the incision complex (Robins *et al.*, 1991). The rodent-complementing human gene, *ERCC-3*, corrects the repair defect in XP group B cells. It is also postulated to be part of the cellular machinery which recognizes and incises damaged DNA (Weeda *et al.*, 1990a). A defect in such a function, analogous to a *uvrA* or *uvrB* mutant in *E. coli*, for example, is predicted to reduce or eliminate repair capacity, depending on the severity of the mutation. As all XP group A cell strains are incapable of UV lesion repair, it is possible that all represent null mutations in the XP-A locus, and that any partial activity is not manifested as an

XP phenotype. XP-B cells retain a limited ability to repair UV photoproducts. A possible interpretation of this is that complete inactivity of the *ERCC-3* gene product is lethal whereas a restricted amount of activity generates the XP-B phenotype.

The severe inability of the XP group D fibroblast strain to remove cyclobutane dimers (~3% of normal) is also in agreement with results reported by others (Zelle and Lohman, 1979; Weinfeld *et al.*, 1986). Although markedly impaired in their ability to repair cyclobutane dimers, XP group D cells retain some ability to excise (6-4) photoproducts (~40% of normal in this study). Mitchell (1988), after studying the same XP group D cell strain (CRL1157), has suggested that XP group D cells might be able to excise some (6-4) photoproducts. Although the repair capacity measured by their radioimmunoassay appears to be about half (20% of normal over a 24 hr period) of that estimated by our method, it should be kept in mind that the former assay scores excision of a given lesion by the time-dependent reduction of its incidence in genomic DNA. This type of approach requires the subtraction of one large number from another and is therefore more susceptible to error.

The failure of XP group D cells to excise additional (6-4) photoproducts after 6 hr post UV implies that some (6-4) photoproducts (~60%) represent a specific class or chromosomal location not acted on by the repair machinery (i.e. domain-specific repair). However, it is not apparent what specific chromosomal domain would constitute 40% of the genome and be selective for excision repair complex entry. An alternative possibility is that productive incision / excision of a fraction of the (6-4) photoproducts occurring in the genome of a UV-irradiated cell precludes the repair of the remainder. This would occur if some limiting component of excision repair was unable to recycle after its initial participation in an incision event. This limiting component, synthesized in catalytic amounts, could constitute the XP-D gene product or, alternatively, an associated protein whose disassociation from the now unproductive repair complex required the action of the XP-D protein. Some insight into this postulate could be achieved by assessing how the extent of repair varies over a range of UV doses.

The XP-D gene has recently been equated to *ERCC-2* (Arrand *et al.*, 1989; Tanaka *et al.*, 1990), and by inference, is postulated to be a helicase (Sung *et al.*, 1987a; Sung *et al.*, 1987b; Harosh *et al.*, 1989). It is plausible that a helicase may be synthesized in rate limiting quantities. Furthermore, whereas a null mutant might be lethal, an altered form of this enzyme may be able to load onto the incised DNA, displace a photolesion-containing excision fragment, but be unable to disassociate and, therefore, incapable of participating in a second repair event. The partial proficiency of (6-4) photoproduct removal, given the complete defect in cyclobutane dimer repair, may reflect the difference in kinetics with which these two UV lesions are excised.

The XP group E fibroblast strain employed in this study consistently showed normal cyclobutane dimer removal, in contrast to reports claiming only 60% UV endonuclease site removal (Zelle *et al.*, 1980). Furthermore, although given sufficient time (24 hr) this strain was proficient at repairing (6-4) photoproducts, the rate was markedly reduced from that in normal cells, in agreement with Roza *et al.* (1990). Whereas normal cells had repaired 100% of these lesions by 6 hr, XP group E cells had removed only 33%. This too, is in conflict with repair estimates utilizing a radioimmunoassay (Mitchell, 1988a). Again, this likely reflects the inherent problems with a technique which monitors the difference between two large numbers, as is also a potential problem when using the enzymatic digestion/HPLC methodology on genomic DNA isolated from post-UV incubated cells (see Chapter 3, page 72). The slower repair of (6-4) photoproducts may account for the delay in recovery from inhibition of DNA synthesis (R. Mirzayans, personal communication), if (6-4) photoproducts are in fact replication blocking lesions (Dahle *et al.*, 1980; Konze-Thomas *et al.*, 1982). In this way, the cell may be buying extra time until these cytotoxic lesions are removed. These results do not, however, translate into functional support for the previously observed lack of a pyrimidine dimer binding protein in XP group E cells (Chu and Chang, 1988).

As had been predicted for XP group F cells, they were totally inept at (6-4) photoproduct repair and yet showed normal proficiency for cyclobutane dimer removal. Given that (6-4) photoproducts are repaired very quickly and to completion in normal cells, and that cyclobutane dimer removal is steady but at a much reduced rate, this easily accounts for previous UDS studies (Zelle *et al.*, 1980; Fujiwara *et al.*, 1985). This earlier work demonstrated that although UDS is very low in group F cells shortly after UV repair (up to 6 hr), it increases after this point at the same rate as in normal cells. Thus, XP-F cells are lacking the fast repair component, but maintain the slow repair component. As (6-4) photoproducts have been removed by 6 hr post UV in normal cells, the slow repair component should be entirely due to cyclobutane dimers. Since XP group F cells carry out this slow component, and since the slope of this part of the repair curve is identical to normal cells, this suggests that these cells are competent at the repair of cyclobutane lesions. This inference has been confirmed by this study. However, it is not possible to explain why other studies have routinely found only 70% UV endonuclease site removal (Zelle *et al.*, 1980) nor why microinjection of *M. luteus* or T4 endonuclease increases

UDS (de Jonge *et al.*, 1985; Yamaizumi *et al.*, 1989). There may either be variation between members of the XP-F complementation group. Alternatively, it is possible that experimental error or variation has been deemed significant, or the purity of the *M. luteus* and T4 UV endonuclease enzyme preparations are in question. Of course, the chance that the XP-F strain used in this study has reverted for a defect in cyclobutane dimer removal cannot be ignored and is not unprecedented (Cleaver *et al.*, 1987). This seems highly unlikely, however, given the excellent correlation of the result found in this study with UDS studies done by two independent groups (Zelle *et al.*, 1980; Fujiwara *et al.*, 1985).

As summarized in Chapter 2, page 34, within the first 6 hr following UV irradiation with 40 J/m^2 of 254-nm light, cyclobutane dimers accounted for only ~20% of all excised lesions in a normal cell. Assuming that the repair of both classes of lesions induces a repair-patch of similar size (Huang *et al.*, 1992), these results indicate that cyclobutane dimers would contribute to only 20% of the total UDS at 6 hr after UV exposure, and (6-4) photoproducts would contribute the remaining 80%. Based on this assumption, expected UDS at 6 hr post UV, for the various XP complementation groups, has been calculated (see Table XII). Although UDS is typically measured at 2 hr post UV, calculations are based on 6 hr repair since: (i) the contribution by each lesion type appears to be in the same proportion at earlier repair times; and (ii) the scarcity of excision fragments at very early times limits the accuracy of such measurements. The calculated values presented in Table XII are within the range of observed UDS values in all cases.

In most instances, the observed UDS values correlate well with the repair capacity predicted by UV endonuclease site removal. The two intriguing exceptions are XP groups D and F. In this study the almost complete defective cyclobutane dimer repair of XP group D cells has been confirmed, and furthermore a capacity to excise ~40% of the (6-4) photoproducts repaired by normal cells in 6 hr, has been demonstrated. Therefore, the level of UDS in XP group D cells, at that time, is estimated to be 33% of normal (i.e., 80% of 40% + 20% of 3%). This value is typical for XP group D cells (Paterson *et al.*, 1984). That the residual level of UDS in XP group D cells is the result of DNA repair synthesis occurring at sites containing a (6-4) photoproduct seems to be, therefore, a reasonable explanation for the apparent discrepancy between their inability to excise cyclobutane dimers (illustrated by the marker deficiency to eliminate UV endonuclease- sensitive sites) and their capacity to carrying out a substantial amount (20-55% of that performed by normal cells) of UV-stimulated DNA repair synthesis (UDS) (Zelle and Lohman, 1979; Paterson *et al.*, 1981). This finding would then also be fully consistent with the observation that microinjected photolyases are unable to reduce the residual level of UDS in XP group D cells and that T4 *denV* gene leads to only partial compensation of the genetic defect in these cells (the transfected cells showing intermediate UV sensitivity) (Arrand *et al.*, 1987; Valerie *et al.*, 1987). Since the *denV* gene product is specific for cyclobutane dimers (Gordon and Haseltine, 1980; Bockrath *et al.*, 1988), it can only substitute for abnormal repair of these photodimers. Knowing that: (i) (6-4) photoproducts are not recognized by this prokaryotic enzyme; and (ii) ~60% of the (6-4) photoproducts are not repaired in XP group D fibroblasts, normal cellular response to the lethal effects of UV light should not be achieved by transfection with the *denV* gene. This conclusion suggests that a minor UV-induced DNA adduct, like a (6-4) photoproduct, may exert a major biological response. Additionally, the ability of XP group D cells to excise a substantial number of (6-4) photoproducts may help to explain the perplexing observation that these cells are much less deficient at excising angelicin adducts from DNA relative to their defect in removing cyclobutane dimers (Cleaver and Gruenert, 1984). Our results suggest that angelicin adducts may be recognized by the same lesion-incising enzyme as are (6-4) photoproducts.

The observed UDS for XP group F cells is substantially lower than their ability to repair (6-4) photoproducts. The calculated UDS, however, based on the excellent ability of these cells to repair cyclobutane dimers along with their inability to remove (6-4) photoproducts (20% of 100% + 80% of 0% = 20%) correlates well with reported values (Zelle *et al.*, 1980; Fujiwara *et al.*, 1985). Thus, as is the case for group D cells, the different kinetics with which the two lesion types are repaired, and the relative efficiency of removing these adducts, explains the apparent discrepancy found between cyclobutane dimer elimination and repair synthesis. Furthermore, this clearly demonstrates that the biphasic curve produced upon studying accumulated UDS in post-UV incubated normal cells is the result of the independent repair of two different groups of UV photoproducts, the fast component largely reflecting (6-4) photoproduct repair, and the slow component exclusively manifesting cyclobutane dimer removal.

Although there is no discrepancy between UDS and UV endonuclease site removal for XP group E as is the case for XP groups D and F, our results indicate that the reduced kinetics with which (6-4) photoproducts are repaired in XP-E cells, and not a deficiency in cyclobutane dimer removal, accounts for subnormal UDS. Thus, to recapitulate the conclusion drawn from XP groups D and F, UDS cannot be taken to correspond totally to the repair of any given lesion type.

Of the six XP complementation groups capable of at least some cyclobutane dimer removal (B, C, D, E, F, and variant), all displayed a distribution of intact and modified lesions similar to that seen in excision fragments derived from post-UV incubated normal cells (data not shown). This indicates that in none of these instances, is a loss of intradimer phosphodiesterase (IDP) activity the cause of the UV sensitivity. XP variant cells are defective in postreplication repair (Boyer *et al.*, 1990). Given the operational definition of postreplication repair as replicative bypass of lesions in the DNA template, it would appear that intradimer phosphodiester cleavage is not, by itself, responsible for this particular cellular process, even though involvement in replicative bypass (in some manner) is a hypothesized function for IDP (see Chapter 2).

In summary, data presented in this study support the longstanding assumption that various assays for measuring the repair proficiency of a mammalian cell reflect distinct repair events. Two widely used techniques for monitoring nucleotide excision repair, namely loss of UV endonuclease-sensitive sites and UDS, do not measure the same phenomenon since: (i) the loss of *M. luteus* or phage T4 UV endonuclease-sensitive sites is a measure of a cell's ability to eliminate only cyclobutane dimers, whereas UDS is the result of the excision repair of various DNA lesions; (ii) UDS depends not only on the number of excised lesions, but also on the length of the repair patch which may vary with the lesion; and most importantly; (iii) the two assays do not relate to the same repair period in most studies. Usually UDS is determined over the first 1 - 2 hr after irradiation, whereas accurate measurements of the disappearance of UV endonuclease-sensitive sites requires much longer incubation times. Therefore, since (6-4) photoproducts are repaired much faster than cyclobutane dimers, UDS, at early times, reflects more the cell's ability to excise (6-4) photoproducts than cyclobutane dimers.

The unique abilities of most XP complementation groups to repair (6-4) photoproducts and cyclobutane dimers (the exceptions being groups A and G, each of which has no capacity to excise either lesion) lends further support to the idea that there must be at least two repair mechanisms operating on UV-induced damage in human DNA. (The other observations supporting this conclusion are summarized on page 73.) The ability to eliminate one type of damage from the genome appears to be independent of the repair of the second. These results contradict the idea that the rapid rate of (6-4) photoproduct removal reflects a higher affinity of a limiting recognition/incision complex for these adducts as compared to cyclobutane dimers (Jones *et al.*, 1992). A reduced level of this enzyme would initially affect only cyclobutane dimer repair, and subsequently, with further reduction, (6-4) photoproduct removal. However, XP group F cells, for example, appear to be normal for cyclobutane dimer excision while being totally inept at (6-4) photoproduct removal.

An alternative explanation has been proposed to account for the phenotype of XP group A revertants (Jones *et al.*, 1992). Although XP group A cells have no ability to repair either cyclobutane dimers or (6-4) photoproducts, their derived UV-resistant revertants regain normal (6-4) photoproducts repair, while remaining incapable of excising cyclobutane dimers. This partial gain in function has been attributed to an alteration in the recognition site of the incision complex such that it has affinity only for (6-4) photoproducts. However, this would imply that each XP complementation group has a different modification at this site, as each has a distinctive repair phenotype with regards to these two major photoproducts. A more plausible explanation is that, although DNA containing (6-4) photoproducts or cyclobutane dimers may be incised by the same enzyme complex, the recognition proteins for these two adducts are different. These may occur simultaneously on the same incision complex, or alternatively, only one or the other may be present on any given multiprotein complex.

The method used in this study reveals only excision from the genome as a whole without indication of repair in discrete regions. In CHO cells, preferential removal of cyclobutane dimers from transcriptionally active genes has been proposed to account for the high UV survival of these cells given their poor overall ability to remove these lesions (Bohr *et al.*, 1985). The implication of this is that UV sensitivity correlates with loss of repair in only very limited regions of the genome rather than total repair. However, the rapid removal of (6-4) photoproducts compared to cyclobutane dimers has been used as evidence by others to demonstrate that these photolesions are the biologically important lesion, with UV survival correlating with overall (6-4) photoproduct removal (Mitchell *et al.*, 1985). There is further evidence for this latter theory. XP group A revertants, as referred to above, display normal UV resistance and have regained the ability to repair (6-4) photoproducts while remaining inept at cyclobutane dimer removal. Studies on preferential repair in these cell strains indicate that it does not occur (Cleaver *et al.*, 1991), questioning the biological importance of this phenomenon. Furthermore, as will be discussed in more detail in chapter 8, members of our laboratory have demonstrated the transient appearance of dimers lacking an intralesion phosphodiester bond in an active gene of normal human fibroblasts (unpublished data). These results, along with results presented in this dissertation, imply that preferential repair may, in fact, be dimer modification. Therefore, measurement of overall repair, as had been achieved here, may accurately reflect UV survival.

cell strain	repair (% of normal) [taken from Kraemer (1983)]		repair proficiency (% of normal at 6 hr post UV) (this study)		
	UV endonuclease site removal	observed UDS	cyclobutane dimer removal	(6-4) photoproduct removal	calculated UDS *
normal (GM38)	100	100	100	100	100
XP-A (CRL1223)	0	0 - 5	0	0	0
XP-B (CRL1199)	< 10	3 - 7	1	10	8
XP-C (CRL1166)	15 - 35	10 - 25	20	11	13
XP-D (CRL1157)	0	20 - 55	3	40	33
XP-E (CRL1259)	60	40 - 60	100	33	46
XP-F (GM3542)	70	10 - 20	100	0	20
XP-G (GM3021)	0	< 5	0	0	0
XP-VARIANT (CRL1162)	100	100	100	100	100

TABLE XII Correlation between observed and expected UDS calculated using the enzymatic digestion/ HPLC assay. * Based on the finding that ~20% and 80% of the excised lesions at 6 hr post UV in GM38 (normal) represent cyclobutane dimers and (6-4) photoproducts, respectively. Assuming that the correction of both classes of lesions results in repair patches of similar size, the amount of UDS expected for the different XP cells at this time is calculated as follows: [20% contribution to UDS by cyclobutyl dimer repair x % proficiency of particular cell strain] + [80% contribution to UDS by (6-4) photoproduct repair x % proficiency of cell strain] = calculated UDS (% of normal). Although UDS is classically measures after 2 hr post-UV incubation, the relative contribution to UDS by the two lesion types is about the same at 2 and 6 hr.

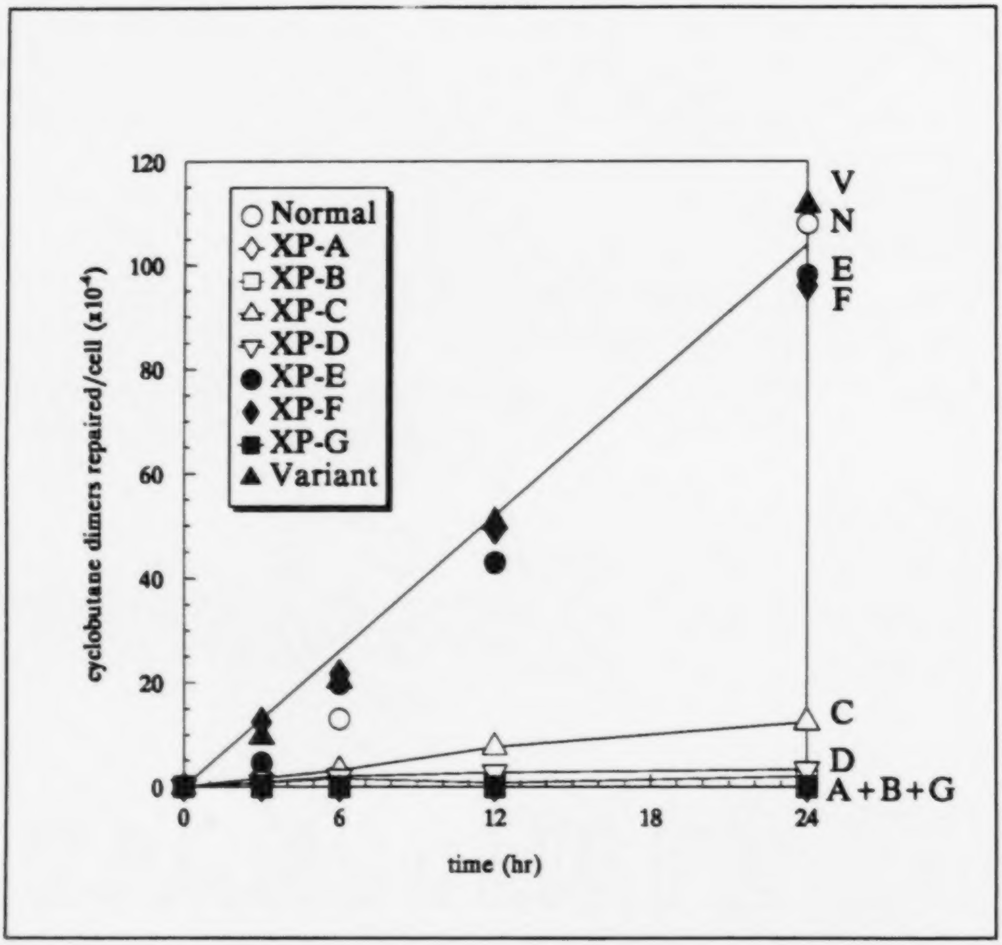


FIGURE 17 Repair of cyclobutane dimers in human (normal and various XP) cell strains following 40 J/m^2 of 254 nm light. C^C removal is calculated as T^C removal x 0.24 (Weinfeld *et al.*, 1986).

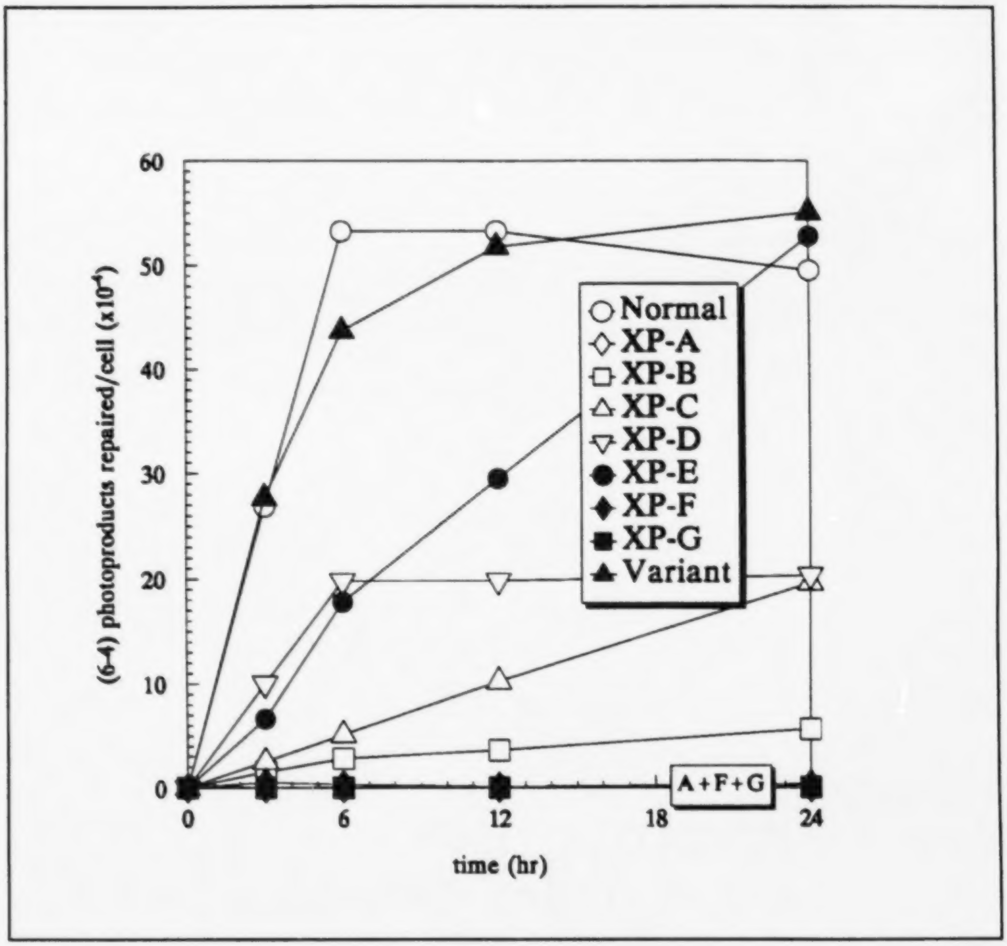


FIGURE 18 Repair of (6-4) photoproducts in human (normal and various XP) cell strains following 40 J/m^2 of 254 nm light. C[p]C excision is calculated as 0.27 x (d-T[p]C + d-T[p]T) (Brash and Haseltine, 1982; Brash *et al.*, 1987; see page 25, 276 for details).

CHAPTER FIVE - THE REPAIR OF UV PHOTOPRODUCTS IN CULTURED FIBROBLASTS DERIVED FROM TRICHOTHIODYSTROPHY PATIENTS AND OTHER UV-SENSITIVE HUMAN SUBJECTS.

INTRODUCTION

Xeroderma pigmentosum is not the only human disorder which is associated with increased sensitivity to ultraviolet light. Another well-documented example is trichothiodystrophy (TTD), an autosomal recessive disorder whose most consistent diagnostic feature is low sulphur content of the hair (Pollitt *et al.*, 1968; Baden *et al.*, 1976; Price *et al.*, 1980). Severe photosensitivity has been reported for about one-half of TTD patients. The remaining one-half exhibit no adverse effects upon exposure to UV light. Investigation of four photosensitive Italian TTD patients (Stefanini *et al.*, 1986) indicated a deficiency in repair of UV-induced DNA damage. Furthermore, cell fusion studies with cells from the different XP complementation groups divulged that the excision repair defect was shared with XP-D cells. Patients who lack photosensitivity show no such repair defect.

The three TTD cell strains (TTD1B1, TTD1GL, and TTD2GL) presented in this study are believed to represent the three classes of TTD and their various responses to UV irradiation have been previously examined in some detail (Lehmann *et al.*, 1988; Broughton *et al.*, 1990). Although none of the original patients displayed photosensitivity, the corresponding cell strains displayed a diverse range of responses to UV. TTD1B1 is a class 3 strain and, following UV irradiation, exhibits completely normal survival, sister chromatid exchange, and mutation rate. UV endonuclease site removal is normal, but UDS is low (40% of normal after 10 J/m^2). This cell strain does not complement the Italian TTD strains which, in turn, do not complement XP-D cells. In contrast, the class 1 strain, TTD1GL, does complement the Italian repair-deficient TTD strains. It is normal in every aspect following UV exposure. TTD2GL (class 2), like TTD1B1, is incapable of complementing XP group D cells. However, it displays abnormally low UV survival, UV endonuclease site removal, and UDS. Phenotypically, it is said to be the most XP group D-like (Broughton *et al.*, 1990). To explain the variable phenotype of TTD, it has been suggested that the TTD gene lies next to the XP-D gene, and that the heterogeneity in DNA repair reflects the length of a deletion which may or may not extent into the XP-D gene to varying degrees, but different mutations in the same gene may be a more reasonable explanation (Lehmann *et al.*, 1988). It has also been proposed that XP group D cells and the non-complementing TTD strains may harbor defects in the same (XP-D) gene, but that the full manifestation of the XP defect simultaneously requires a natural killer cell defect (Norris et al., 1990).

It is surprising that the excision repair-deficient TTD strains (classes 2 and 3) fail to complement XP-D cells as their repair characteristics are distinctly different. The class 2 strain, TTD2GL, displays essentially no UDS following UV irradiation, yet XP group D cells classically show 20-55% of normal UDS. Alternatively, TTD1B1 (class 3) has low UDS in the range of XP-D cells, but a time course repair replication study (Lehmann *et al.*, 1988) yielded a curve that was very XP group F-like [i.e it lacked the fast-repair component which immediately follows irradiation but appeared to be normal for the slow repair component (Zelle *et al.*, 1980; Fujiwara *et al.*, 1985)]. With the aim of shedding some light on these discrepancies, the three TTD strains described above were analyzed for their ability to remove both cyclobutane dimers and (6-4) photoproducts using the three-stage enzymatic digestion/HPLC procedure described in Chapter 2 (beginning page 14), on excision fragments derived from post-UV incubated cells.

In addition, two previously undescribed human fibroblast strains, BD-1 and CVT, derived from patients showing photosensitivity but lacking any defined disorder, were also included in this analysis.

EXPERIMENTAL PROCEDURES

For more detailed experimental procedures, see Appendix A, page 254.

Cell and culture conditions. Experiments were conducted on the following five human diploid fibroblast strains: three strains (TTD1B1, TTD1GL, and TTD2GL) established from skin biopsies of patients diagnosed with trichothiodystrophy; and two strains (BD-1 and CVT) established from skin biopsies of patients showing UV-sensitivities of unknown origin. TTD1B1, TTD1GL, and TTD2GL were supplied by Dr. Alan Lehmann (Sussex University, Brighton, UK). BD-1 was provided by Dr. R. Ramssey (Toronto Hospital for Sick Children, ON). CVT was obtained from Dr. Betsy Hirsch (University of Minnesota, Minneapolis, MN). BD-1 and CVT are previously undescribed fibroblast strains.

BD-1 fibroblasts were established from a 4-year-old white female who was referred to the Toronto Hospital for Sick Children for investigation of unusual hypo- and hyperpigmentation on sun-exposed areas of the skin.

CVT fibroblasts were established from a 6-year-old white male who was referred for evaluation of an unusual rash on sun-exposed areas. The rash was characterized by both hypo- and hyperpigmentation, telangiectasias, and extensive freckling. Analysis by Dr. James E. Cleaver (University of California, San Francisco) established that the UV-sensitivity identified with this cell strain was not associated with the repair deficiency present in any of the seven repair-deficient xeroderma pigmentosum complementation groups, A-G (unpublished data).

All culture conditions for all cell strains were identical to those presented in Chapter 2 (page 14).

Other material and methods. All materials and procedures for (i) [^{3}H]thymidine-labelling of cells, (ii) UV irradiation, (iii) collection of excision fragments, (iv) formic acid hydrolysis, (v) enzymatic hydrolysis, (vi) alkaline hydrolysis, and (vii) reverse-phase HPLC have been described in Chapter 2 (pages 14-18, 273).

RESULTS

Enzymatic digestion/HPLC analysis has previously been employed to quantitate the two major classes of lesions found in the TCA-soluble material derived from post-UV incubated cells. This was extended in this study to include three trichothiodystrophy cell strains (two of which are known to have excision repair defects), and two other human cell strains derived from patients displaying photosensitivity, but with undefined disorders. The absolute numbers of both cyclobutane dimers and (6-4) photoproducts contained within excision fragments, and therefore excised from the DNA, have been calculated for all five cell strains for post-UV incubation times of 0, 3, 6, 12, and 24 hr. Computations are based on the assumptions set out earlier in Tables III and V (see pages 43 and 45), as well as the premise that C^C excision is equal to T^C excision x 0.24 (Weinfeld *et al.*, 1986) and C[p]C repair is equal to (T[p]T + T[p]C) removal x 0.27 (Brash and Haseltine, 1982; Brash *et al.*, 1987).

Cyclobutane dimer repair in the three trichothiodystrophy strains is summarized in Figure 19. (Data for the normal cell strain, GM38, have been included here, as well as in Figures 20-22, to permit direct comparison.) It is readily apparent that the class 1 TTD strain, TTD1GL, showed no defect in cyclobutane dimers removal, repairing a total of 1.08 x 10^6 pyrimidine dimers/cell by 24 hr post UV. In contrast, TTD2GL (class 2) demonstrated no ability to carry out this mode of repair. By 24 hr following UV irradiation, cyclobutane dimers could still not be recovered in the TCA-soluble material derived from this strain. TTD1B1 (class 3) was capable of repairing cyclobutane dimers, but only 3.64 x 10^5/cell, or ~33% of those repaired by either the normal cell strain, or the excision-repair proficient strain, TTD1GL.

Figure 20 summarizes (6-4) photoproduct repair in the same three TTD cell strains. As with cyclobutane dimer repair, TTD1GL had no deficiency in (6-4) photoproduct removal, excising 5.65 x 10^5/cell (or 100% of those induced; see Chapter 2, Table III, page 43) by 6 hr subsequent to UV treatment. TTD1B1 was capable of removing these lesions, but whereas a normal strain had completed this process by ~ 6 hr post UV, TTD1B1 had not accomplished this task until 24 hr following 40 J/m^2, repairing only 1.71 x 10^5/cell (6-4) photoproducts by 6 hr, but all 5.84 x 10^5 by 24 hr. TTD2GL was markedly deficient at (6-4) photoproduct excision, but unlike cyclobutane dimer removal, this mode of repair was not entirely absent [3.3 x 10^4 (6-4) photoproducts/cell (5% of normal excision) by 6 hr post UV and 1.10 x 10^5 (20% of normal) by 24 hr].

The remaining two UV-sensitive cell strains, BD-1 and CVT, displayed the repair characteristics illustrated in Figures 21 (for cyclobutane dimers) and 22 [(for 6-4) photoproducts]. In both strains, cyclobutane dimer removal was defective. Although both cell strains displayed ~60-75% of normal repair capacity (7.6 - 9.6 x 10^4/cell) for these photoproducts after 6 hr of post-UV incubation, by 24 hr the process had been impeded, with only a total of 1.39 x 10^5 and 3.07 x 10^5/cell (~13 and 28% of normal) having been excised in BD-1 and CVT, respectively.

(6-4) photoproduct excision was also deficient in both BD-1 and CVT. BD-1 demonstrated steady but reduced repair, having acted on 3.4 x 10^5/cell (or ~64% of normal) by 24 hr post UV. Like cyclobutane dimer repair, (6-4) photoproduct removal was initiated more rapidly than the residual rate by 24 hr following irradiation. Three hr into post-UV incubation, CVT displayed ~33% of the repair capacity of normal cells (a total of 9.1 x 10^4/cell). However, by 24 hr, the absolute number of excised (6-4) photoproducts had increased to only 2.04 x 10^5/cell, or approximately double.

DISCUSSION

The utility of the enzymatic digestion/HPLC protocol for quantitating UV photoproducts has been demonstrated (Chapter 2) by (i) comparing data acquired through this method with that measured by various other techniques (see page 26) and (ii) for normal human fibroblasts and CHO cells, showing that lesions excised from genomic DNA can be accounted for by their appearance in excision fragments (see pages 26 and 78). Therefore, this methodology has been extended to other UV-sensitive human fibroblast strains with the hope of uncovering some novel primary defect.

Lehmann *et al.* (1988) monitored, in response to 4 J/m^2, the removal of UV endonuclease-sensitive sites in the same three TTD strains studied here. As expanded upon below, some of their results differ substantially from those presented here. However, as noted previously, this methodology draws it conclusions from a technique which requires the subtraction of one large number from another. In addition, this work used a UV fluence 10 times lower than in the present study. Broughton *et al.* (1990) have also explored the repair of (6-4) photoproducts in these same strains, using a radioimmunoassay (Mitchell *et al.*, 1985). However, their study extends only over the first 6 hr of post-UV incubation, and in this way, does not show the extent to which repair can proceed. In the current study, analysis has been assessed over the 24 hr following 40 J/m^2.

The trichothiodystrophy cell strain, TTD1GL, was indistinguishable from normal cells with respect to the repair of both cyclobutane dimers and (6-4) photoproducts, as determined by the enzymatic hydrolysis/HPLC assay system. It likewise was found to accumulate a distribution of intact and modified cyclobutane dimers indiscernible from normal human fibroblasts. This was anticipated, as this class 1 TTD strain displays no adverse reaction to UV irradiation, and has been shown by other laboratories to be proficient at excising both major UV photoproducts (Lehmann *et al.*, 1988; Broughton *et al.*, 1990).

Although TTD1B1 and TTD2GL have been reported to be unable to complement the repair defect in XP group D cells (Lehmann *et al.*, 1988), their deficiencies in the repair of cyclobutane dimers and (6-4) photoproducts, as compared to repair phenotype of XP-D cells, are not consistent with this result. Under the irradiation conditions employed here, TTD1B1 was defective in both cyclobutane dimers and (6-4) photoproduct removal. Others (Lehmann *et al.*, 1988; Broughton *et al.*, 1990) have reported normal cyclobutane dimers excision and have attributed the reduced UDS solely to the 50% reduction in (6-4) photoproduct removal. In the current study, this strain excised only about one-third of the cyclobutane dimers removed by normal cells after 40 J/m^2. Furthermore, although (6-4) photoproduct removal appeared defective at early post-UV repair times, it proceeded to completion by 24 hr post UV. These kinetics of (6-4) photoproduct repair are much more XP group E-like than comparable to the repair prototype of XP group D cells (see Chapter 4, page 90). (Of course the similarity to XP-E is limited as these cells were not deficient in the repair of cyclobutane dimers.) The slower rate of (6-4) photoproduct removal in TTD1B1 has been previously reported by Broughton *et al.* (1990), although over a shorter repair period. Such reduced repair kinetics imply a less abundant repair enzyme, or alternatively, an impeded turnover rate of an otherwise normal enzyme. Also in agreement with Broughton, this reduction in (6-4) photoproduct repair accounts for the low UDS displayed by these cells. However, this concordance is attained because (6-4) photoproduct excision contributes to the majority of repair replication at early post-UV times, and not because this strain is normal in acting on cyclobutane dimers.

The difference between the cyclobutane dimer repair results presented here and those found previously for TTD1B1 (Lehmann *et al.*, 1988; Broughton *et al.*, 1990), may merely reflect a deficiency which is not manifested under low doses of UV-irradiation. This would be predicted if a component of nucleotide excision repair is limiting in this cell strain at higher UV doses. Although quantities may be sufficient to allow seemingly normal UV photoproduct excision at low doses, as the absolute number of lesions increases with dose, a deficiency would be revealed. According to the results of Lehmann *et al.* (1988), TTD1B1, as well as normal cells, remove 60% of the UV endonuclease-sensitive sites by 24 hr following UV irradiation with 4 J/m^2. At an induction rate of 1 x 10^5 cyclobutane dimers/cell/J/m^2 [Paterson *et al.*, 1973; this study (see page 78)], a normal cell has removed 2.4 x 10^5 cyclobutane dimers from its genome in the first 24 hr following a fluence of 4 J/m^2. After 40 J/m^2, a 10-fold increase in the incidence of these lesions appears to increase the rate of excision 4-fold, enabling 1 x 10^6 cyclobutane dimers/normal cell to be excised in this same 24-hr period. This implies that some component of the nucleotide excision process is inducible (i.e. it can be upregulated). In TTD1B1, although the number of cyclobutane dimers removed after 4 J/m^2 in 24 hr was the same as for a normal cell, following 40 J/m^2, the total number had increased only 1.5-fold. Therefore, the cyclobutane dimer excision repair defect in TTD1B1 may be due to a less efficient induction mechanism which normally allows upregulation of some gene product involved in cyclobutane dimer removal with increased

UV fluence. Alternately, this result may simply reflect the fact that the repair process is suboptimal at 4 J/m^2.

In this study, TTD2GL was incapable of repairing cyclobutane dimers after treatment with 40 J/m^2 of 254-nm light. A previous study (Lehmann *et al.*, 1988), demonstrated 20% loss of UV endonuclease-sensitive sites by 24 hr following 4 J/m^2. However, at this latter fluence, 4 x 10^5 cyclobutane dimers are induced in each genome, so that 20% removal would correspond to eliminating 8 x 10^4 of these adducts. If, following 40 J/m^2 (which would introduce 4 x 10^6 dimers/cell), these cells can still repair only 8 x 10^4 dimers, this would correspond to only 2% removal and would not be differentiated from background. This, then, implies that TTD2GL, like TTD1B1, is incapable of increasing the rate of repair of this class of UV lesion with increased dose, as it appears normal cells can.

The defective removal of (6-4) photoproducts in TTD2GL is characteristic of XP group C cells, rather than reminiscent of the non-complementing XP group D cells. Like XP-C, in the 24 hr following irradiation (40 J/m^2), TTD2GL removed only approximately 20% of the (6-4) photoproducts induced, with kinetics very much like those found in XP-C cells (see Figure 18, page 98). The same result was found by Broughton *et al.* (1990), using a radioimmunoassay, at a lower UV fluence (4 J/m^2). This repair phenotype is not unlike that of TTD1B1, except that it is more extreme.

If the results at low UV fluences of others are valid, and have not been affected by the limitations of the measurement procedure used, then cyclobutane dimer repair in both TTD1B1 and TTD2GL appears to be affected only at higher UV doses (Lehmann *et al.*, 1988), whereas (6-4) photoproduct removal is impaired even at low doses (Broughton *et al.*, 1990). Jones *et al.* (1992) have proposed that, in normal cells, the faster rate of (6-4) photoproduct removal compared to cyclobutane dimer repair is due to more efficient recognition of the former class of UV adduct by the sole recognition site of the incision complex. Furthermore, they suggest that a defect in the repair of one or both classes of damage is due to a mutation which alters this lone recognition site. However, the proposal presented in this dissertation, that (6-4) photoproducts and cyclobutane dimers are dealt with by different recognition mechanisms (see page 95), is supported by the seeming independency of repair of these two classes of UV adducts in classes 2 and 3 TTD cell strains (as well as in several of the XP complementation groups; see Chapter 4, Figures 17 and 18). Nevertheless, it is also apparent that these repair pathways converge, as there is abundant evidence indicating that a mutation at a single locus can affect the removal of both cyclobutane dimers and (6-4) photoproducts. It is not yet clear how various bulky lesions are recognized, or how these presumably separate recognition mechanisms subsequently converge into a common incision / excision pathway, but it is obvious that our detailed understanding of nucleotide excision repair in human cells is still lacking and may need to await cloning of all the genes involved in the process.

XP group D cells fail to complement the repair defect present in classes 2 and 3 TTD cell strains, represented in this study by TTD2GL and TTD1B1, respectively. It has previously been reported (Broughton *et al.*, 1990) that the repair characteristics of class 2 trichothiodystrophy are indistinguishable from those exhibited by XP group D cells. However, an assessment of the repair properties of fibroblast strains representing both cell types, using the new enzymatic hydrolysis / HPLC methodology, revealed distinct differences between them. Class 3 TTD cell strains are also phenotypically distinct from XP-D cells with respect to their repair characteristics. In fact, it is simpler to relate class 2 and 3 TTD repair defects, with class 2 being a more severe form of class 3. These observations bring into question the relationship of the XP-D and TTD loci.

To explain the lack of a clear XP-D phenotype in the TTD strains which cannot complement the former defect, as well as the diversity of repair properties between TTD strains, Lehmann *et al.* (1988) have proposed that the TTD and XP-D are different manifestations of defects in the same gene. Although it is difficult to recognize the correlation, these authors point out that diverse clinical symptoms coincide with mutations in the same globin gene. Alternatively, it has been proposed that the mutations in TTD patients with repair deficiencies represent deletions of the TTD locus which extend into the neighboring XP-D gene (Stefanini *et al.*, 1986). Class 2 TTD would then differ from class 3 based on the length with which the deletion extends into this repair gene. As has been proposed in Chapter 4 of this dissertation, if the repair defect in XP group D cells is due to a limiting factor which can initially participate in a productive incision / excision complex, but cannot be recycled for subsequent rounds of active repair function, it is not hard to imagine how a different mutation in the same locus would result in low levels of an otherwise normal protein, yielding a reduced rate of repair as is typical of repair-deficient TTD strains. However, one would then expect to find XP-D cell strains with repair profiles much more like these TTD strains. Although only one XP group D strain has been studied in the current work, the inability of previous investigations to find any substantial repair as assayed by time-dependent disappearance of UV endonuclease-sensitive sites indicates that, unlike class 3 TTD cell strains, XP group D cells are unable to repair a substantial number of cyclobutane dimers. This proposed genomic contiguity is, therefore, challenged.

Whereas XP group D patients display marked photosensitivity and a predisposition to cancer, the patients from which the nonXP group D-complementing TTD cell strains, TTD1B1 and TTD2GL, were derived exhibited no apparent adverse effects upon UV exposure. One recent suggestion to account for this inconsistency proposes that manifestation of the XP phenotype requires two discrete mutations, one affecting the expression or function of an XP gene product, and the second suppressing natural killer (NK) activity (Lehmann and Norris, 1989). Such a pattern of inheritance for XP has been previously predicted (Lambert and Lambert, 1985). This idea is not purely speculation, as depressed natural killer activity is displayed by XP patients but not by those with trichothiodystrophy or Cockayne's syndrome (Norris *et al.*, 1990). Furthermore, XP patients exhibit reduced catalase activity which may be indicative of diminished NK activity (Lehmann and Norris, 1989). Therefore, a repair deficiency, in itself, would not be manifested into a disease state because immune surveillance would not allow mutated cells to survive and propagate. The effect of the repair-deficiency would be cell death, rather than predisposition to cancer. However, this does not account for the lack of photosensitivity in some repair deficient TTD patients. Furthermore, it implies that there are many individuals in the population who are homozygous for a defective XP gene, but who do not exhibit a disease state because of normal natural killer activity. The implications of this are unknown.

A final possibility which warrants consideration is that, although the repair defect in TTD cell strains cannot be complemented by XP group D cells, the affected loci are not the same. This would occur if the associated mutant gene products behaved in a manner antagonistic to the normal gene products, when present in combination, thereby disabling normal repair function. Alternatively, given the sulfur deficiency characteristic of TTD patients, the insufficiency of a sulfur-containing protein may interfere with some essential process in the presence of the XP-D mutation.

The two other UV-sensitive human cell strains included in this study, BD-1 and CVT, each exhibited unique phenotypes with respect to their capacity to remove cyclobutane dimers and (6-4) photoproducts. However, given the difference in repair phenotype of XP group D cells and the noncomplementing TTD strains, it is impossible from this information to conclude whether the mutations in BD-1 and CVT occur in one of the repair genes represented by the seven repair-deficient XP complementation groups, or whether these are previously undetected genes essential for repair of UV-induced DNA damage.

Three of the four repair deficient strains in this study, TTD1B1, CVT, and BD-1, were capable of removing at least a portion of the cyclobutane dimers induced in their genome. In all cases, a normal capacity to sever the intradimer phosphodiester bond of these lesions was observed. A mutation affecting intradimer phosphodiesterase activity is, therefore, not the cause of the repair defect present in any of these strains. The inability to uncover a UV-sensitive mutant which lacks this activity may indicate that (i) intradimer phosphodiesterase (IDP) is an essential function or (ii) the loss of this activity is not manifested as a UV-sensitive phenotype. However, although several UV-sensitive cell strains have been studied [including human (Chapter 4 and this study), CHO (Chapter 3), and *E. coli* (Chapter 7)], all possibilities are far from being exhausted (see Chapter 9).

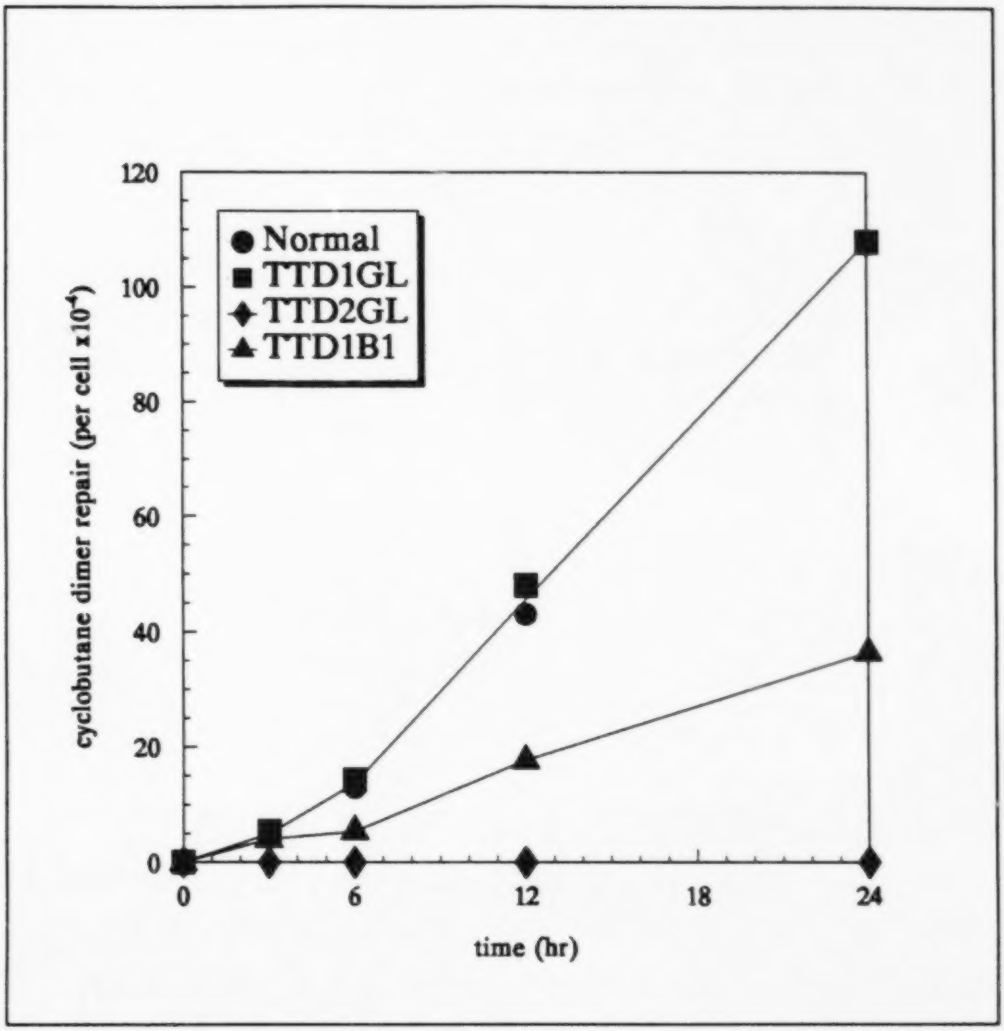


FIGURE 19 Repair of cyclobutane dimers, following exposure to 40 J/m^2 of 254-nm light, in three trichothiodystrophy cell strains and one normal human (GM38) strain. Data for GM38 have been taken from Table V, Chapter 2 (page 45). The number of T^T and T^C dimers excised per cell is based on the assumptions set forth in Tables III and V (pages 43 and 45). The total number of dimers repaired has been determined by the numbers of T^T + T^C found in excision fragments + 0.24 x T^C content (the calculated C^C content).

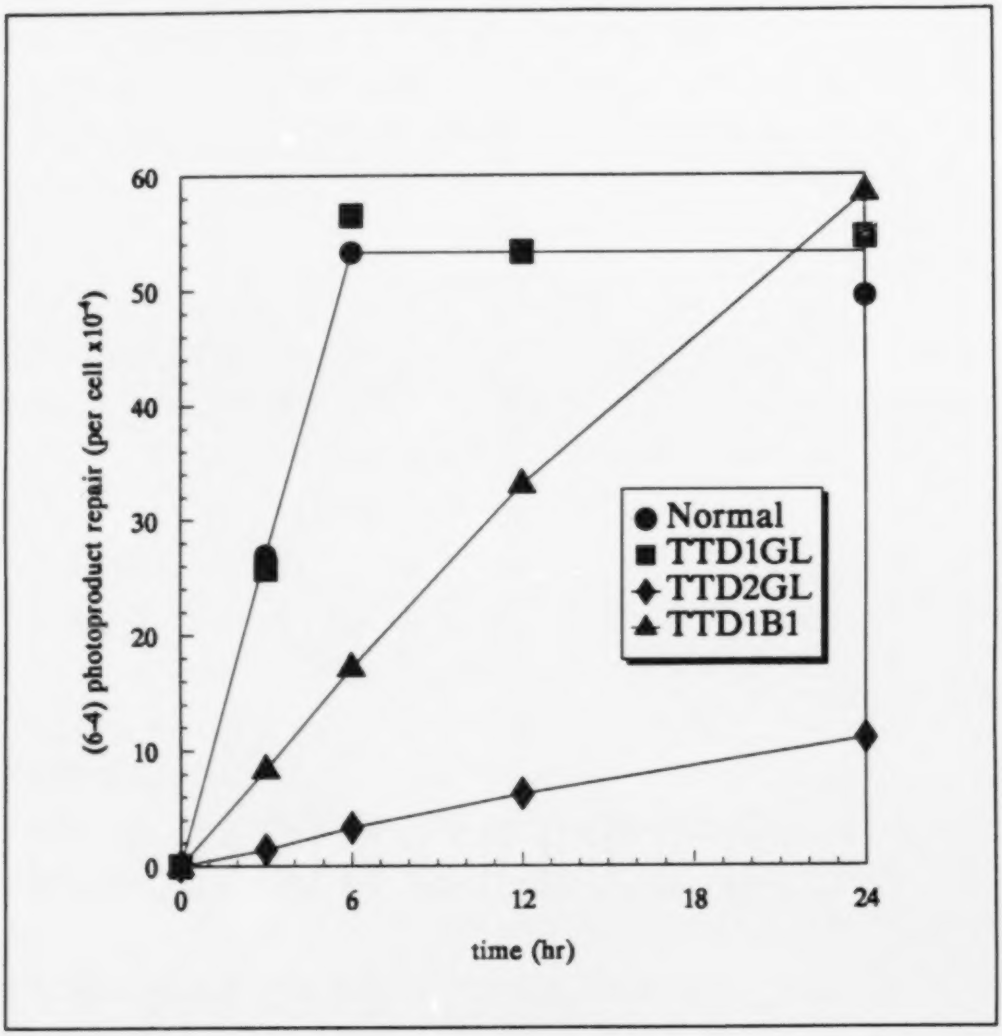


FIGURE 20 Repair of (6-4) photoproducts, following 40 J/m^2 of 254-nm light, in three trichothiodystrophy cell strains and one normal human (GM38) strain. Data for GM38 have been taken from Table V, Chapter 2 (page 45). The total number of (6-4) photoproducts repaired has been determined by the d-T[p]T + d-T[p]C content of excision fragments + 0.27 x this value (the calculated C[p]C content; see text for details).

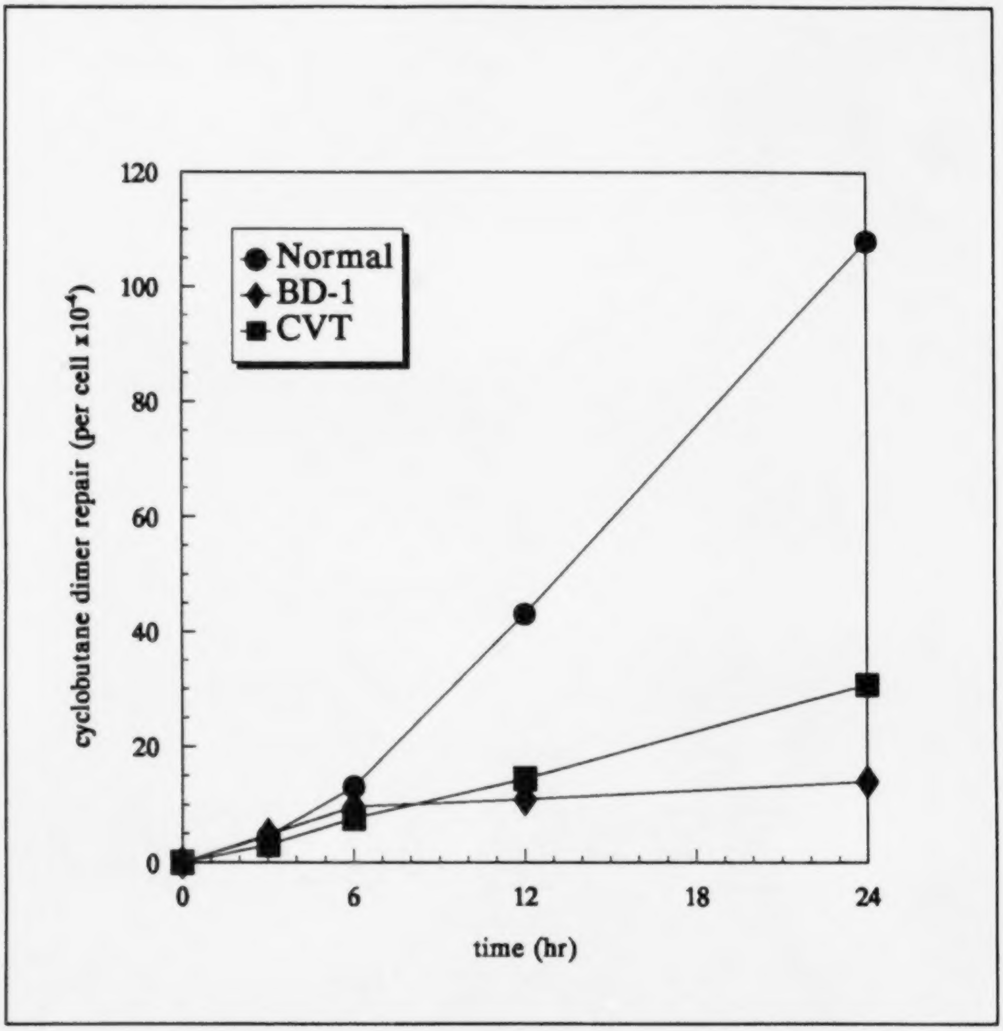


FIGURE 21 Repair of cyclobutane dimers in two human cell strains, BD-1 and CVT, derived from patients displaying UV sensitivity. Results for the normal human fibroblasts strain, GM38, have been redrawn for comparison. Values have been calculated in a manner analogous to that given in Figure 19.

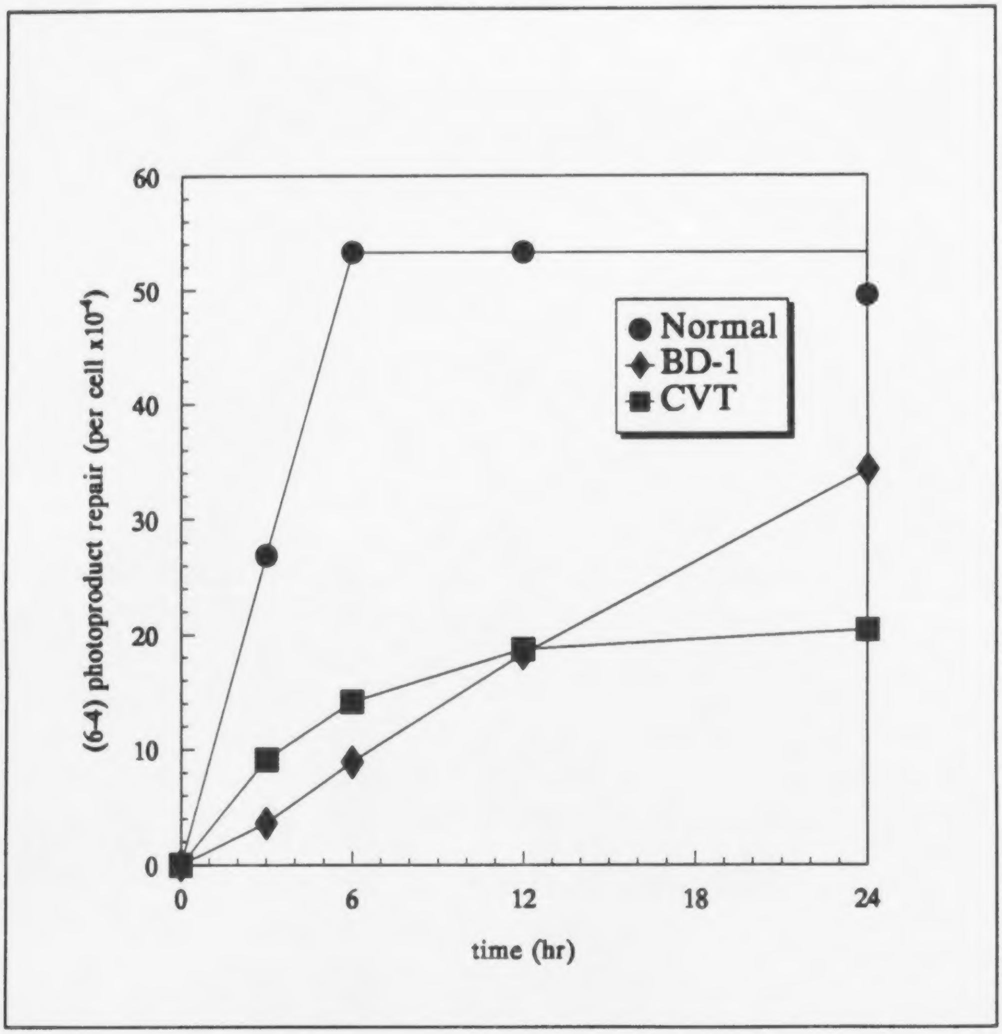


FIGURE 22 Repair of (6-4) photoproducts in two human cell strains, BD-1 and CVT, derived from patients displaying UV sensitivity. Results for the normal human fibroblasts strain, GM38, have been redrawn for comparison. Values have been calculated in a manner analogous to that given in Figure 20.

CHAPTER SIX - THE REPAIR OF A NOVEL UV-INDUCED DNA LESION, THE TA* PHOTOPRODUCT, IN NORMAL AND XP HUMAN FIBROBLASTS

INTRODUCTION

For many years, cyclobutane dimers, the major lesions occurring in UV-irradiated DNA, were considered to be the biologically relevant UV photoproduct. Although (6-4) photoproducts had been known for some time (Johns *et al.*, 1964) it was some time until this lesion, which occurs at ~10% the frequency of cyclobutane dimers, was recognized as having biological significance, and are currently considered the major cytotoxic lesion (for review see Mitchell and Nairn, 1989), although the significance of cyclobutane dimers cannot be ignored (Broughton *et al.*, 1990). There still remain, however, some UV-sensitive cell strains without a defect in their ability to excise either cyclobutane dimers or (6-4) photoproducts. One notable example is the XP variants.

Although XP variant cells are presumed to be defective in postreplication repair (Boyer *et al.*, 1990), it is has recently been hypothesized that their defect lies in the repair of a noncyclobutane dimer lesion (Francis and Regan, 1986; Francis *et al.*, 1988). However, (6-4) photoproduct removal is normal in these cells (Mitchell and Nairn, 1987; this dissertation, Chapter 4).

Another minor UV photoproduct has been recently described, the TA* photoproduct, involving the dimerization of a thymine to an adjacent adenine (Bose *et al.*, 1983; Bose *et al.*, 1984; Bose and Davies, 1984; Kumar and Davies, 1987). Its biological significance is currently unknown. Induced at presumably a very low frequency with 254-nm light [estimated at about 1% the frequency of cyclobutane dimers (Bose *et al.*, 1983; Bose and Davies, 1984)], there has been no assay available to follow their removal from post-UV incubated cells. Thus it seemed reasonable to adapt the enzymatic hydrolysis methodology, outlined in Chapter 2, to explore some basic repair characteristics of this rare lesion, and to ascertain its repair status in XP variant cells.

TA* lesions are repaired rapidly in normal human cells, with kinetics almost identical to the removal of (6-4) photoproducts. Moreover, XP variant cells are not deficient in the repair of this lesion, thus eliminating another minor photoproduct whose persistence in the DNA would explain the defect in these cells. In addition, studies in XP complementation groups A through G demonstrate that, even given a partial or complete (6-4) photoproduct repair deficiency, TA*

lesions are always repaired to the same extent, and with the same kinetics, as are (6-4) photoproducts. This suggests that although data support separate repair pathways (or at least recognition mechanisms) for each cyclobutane dimers and (6-4) photoproducts, TA^* photoproducts are identified by the same recognition protein and/or repair pathway as are (6-4) photoproducts.

EXPERIMENTAL PROCEDURES

For more detailed experimental procedures, see Appendix A, page 254.

Identification of the TA* photoproduct. The alternating copolymer, d(AT)$_n$ was purchased from Sigma Chemical Company (St. Louis, MO). Opposite strand synthesis was carried out, basically by the method of Efstratiadis *et al.* (1976) for cDNA second strand synthesis, utilizing 1 µg of d(AT)$_n$, in the presence of 20 units of DNA polymerase I, 5 µCi/ml [^{3}H]-TTP (1 mCi/ml; 41 Ci/mmol; NEN Canada, Montreal, PQ), 50 µM unlabelled TTP, and 1 mM unlabelled dATP, for 20 hr at 20°C, to produce a radioactively labelled substrate (170 dpm/pmol thymine) for further analysis. 200 µl of the resulting 214 µM [^{3}H]-labelled d(AT)$_n$ was mixed with 800 µl of ddH$_2$0 in a 40-mm Petri dish (Lux Scientific Corp., Newbury, CA) and irradiated with a total fluence of ~10 kJ/m^2 of 254 nm light (for source, see page 15, 258). After digestion overnight with staphylococcal nuclease, DNase I, snake venom phosphodiesterase, and calf alkaline phosphatase under previously described conditions (see page 15, 263, 274) the reaction mixture was dialysed overnight, using 3 kDa MW cut-off dialysis membrane, (see page 15) against 2 *l* H$_2$O. One-half of the retained material was analyzed by reverse-phase HPLC (see page 17). The second aliquot was first digested with nuclease P1 and calf alkaline phosphatase (see page 17, 269) and subsequently analyzed by the same procedure. The trinucleotide, d-ApT{p}A, is retained until fraction 58. The dinucleotide, d-T{p}A, elutes at fraction 48.

Cell strains and culture conditions. Experiments were conducted on nine diploid fibroblast strains established from skin biopsies of human subjects: GM38 (normal), CRL1223 (XP group A), CRL1199 (XP group B), CRL1166 (XP group C), CRL1157 (XP group D), CRL1259 (XP group E), GM3542 (XP group F), GM3021 (XP group G) and CRL1162 (XP variant). The sources of these strains have been described in Chapters 2 (page 14) and 4 (page 86, 254). All culture conditions were identical to those presented in Chapter 2 (page 14).

Subsequent material and methods. All materials and procedures for (i) [^{3}H]thymidine-labelling of cells, (ii) UV irradiation, (iii) isolation of genomic DNA, (iv) preliminary digestion of lesion containing DNA, and (v) dialysis of resulting trinucleotides and mononucleosides have been described in Chapter 2 (beginning page 14).

Purification of trinucleotides by DEAE-cellulose and nuclease P1 digestion. The trinucleotides retained in the dialysis membrane were subjected to a second enrichment procedure. A 2-ml DEAE-cellulose column (Whatman DE 23, Whatman Inc., Clifton, NJ) was used to remove the remaining free thymidine present. This column was first equilibrated with 10 mM ammonium acetate (pH 7.0), and the hydrolysate subsequently applied at a flow rate of 10 ml/hr. After washing the column with 10 ml equilibration buffer to eliminate the free mononucleosides, the oligonucleotides were eluted at the same flow rate with 10 ml 0.4 M ammonium acetate (pH 7.0). Eluted fractions containing radioactivity were pooled, evaporated under reduced pressure, redissolved in 1 ml of ddH_2O, and desalted using a Waters SEP-PAK C_{18} cartridge (Waters Associates, Mississauga, ON). The DEAE-cellulose column was a necessary addition to the protocol as d-T{p}A elutes after the often immense peak of thymidine. This additional purification step allowed a much larger proportion of the free thymidine to be removed than by dialysis alone, thus allowing more accurate quantitation of the TA* lesion. The desalted trinucleotides were subsequently hydrolysed with nuclease P1 and calf alkaline phosphatase, as described previously (page 17, 269), and analyzed by reverse-phase HPLC (see page 17).

Measurement of the rate of induction of TA* lesions in genomic DNA. One plate of [^{3}H] thymidine-labelled GM38 cells was subjected to each of the following fluences of 254 nm light: 0, 25, 50, 100, or 250 J/m^2. Cells were stored on ice immediately after irradiation. Genomic DNA was isolated, enzymatically digested, and dialysed, and the isolated trinucleotides were further concentrated using a DEAE-cellulose column, and treated with nuclease P1 as described above. The TA* lesions present, as well as (6-4) photoproducts and cyclobutane dimers, were quantitated by HPLC analysis.

RESULTS

Identification of the TA* photoproduct. Since the only base neighboring a labelled thymidine in d(AT)$_n$ substrate is an adenine, the only labelled dimer induced by 254-nm irradiation should be the TA* adduct. The UV treatment (10 kJ/m^2) was followed by (i) enzymatic digestion, to yield lesion-containing trinucleotides and mononucleosides, and (ii) dialysis, to concentrate the trinucleotides by reducing the amount of mononucleosides present. About 1.1% of the radiolabel was recovered after dialysis. Subsequent HPLC analysis of this material is shown in Figure 23A and revealed that 54.4% of the radioactivity eluted at fraction 58. This did not correspond to any other previously identified thymine-containing species, and presumably represented the TA*-containing trinucleotide, d-ApT{p}A. As expected, a peak (9.3% of the total radioactivity) was found to migrate at the retention time of thymidine, and in addition, a small amount (6.6% of total radiolabel) of thymine was present. The remainder of the radiolabel was distributed throughout the chromatogram in small unidentified peaks, and perhaps represented other minor adducts or radiolysis products.

Nuclease P1/calf alkaline phosphatase digestion of a second aliquot of this same trinucleotide preparation resulted in the HPLC chromatogram illustrated in Figure 23B. The major radioactive peak eluted in a novel position (i.e., fraction 48), and, by inference, was the dinucleotide d-T{p}A. The percentage of total radioactivity representing this new species (58.1%) was, within experimental error, the same as the major peak displayed in Figure 23A (54.4%). It is also evident from Figure 23B, that the small unidentified peaks distributed throughout the chromatogram in Figure 23A, were resolved into thymidine by NP1 digestion.

The rate of induction of TA* lesions by 254-nm light in genomic DNA. Irradiation of cellular DNA with 254-nm light, using fluences up to ~ 5 kJ/m^2, induces (6-4) photoproducts at about 18% the frequency of cyclobutane dimers (see Chapter 2). The number of TA* lesion produced under similar conditions was estimated by exposing normal human fibroblasts (GM38) to various doses of 254-nm radiation and assessing the lesion content using a variation on the enzymatic hydrolysis (see Experimental Procedures). As the cyclobutane dimers and (6-4) photoproducts could be quantitated in the same experiment, they served as an internal control. The numbers of T^T, T^C, T[p]Py, and TA* lesions induced in the genomic DNA of GM38 following various doses of 254-nm radiation (ranging from 0 to 250 J/m^2) are summarized in Figure 24. These values were calculated as the (% of total radiolabel retained after dialysis) x (% of radioactivity retained by DEAE-cellulose) x (% of label in the purified trinucleotides corresponding to the species of interest, as determined by HPLC analysis) x [the number of thymidine residues present in the human genome as calculated in the legend of Table III (see page 43)]. The raw data accumulated for these various intermediate procedures have not been included for simplicity. Figure 24 illustrates that over the range of fluences studied, TA* photoproduct induction is linear. Lesions were induced at the rates of 4.1 x 10^4 T^T, 3.0 x 10^4 T^C, and 1.1 x 10^4 T[p]Py per cell/J/m^2. The excellent agreement of these values with published results (Paterson *et al.*, 1973; Mitchell *et al.*, 1985) provides confidence of the accuracy of the method.

The number of TA* lesions found in genomic DNA following fluences of 25, 50, 100, and 250 J/m^2, under the conditions employed here, were 1.5 x 10^5, 2.5 x 10^5, 7.0 x 10^5, and 1.21 x 10^6, respectively. Given an induction rate of 1.4 x 10^4 for all (6-4) photoproducts (i.e. including C[p]C lesions; see page 25, 276), TA* lesions occurred at ~35% the frequency of (6-4) photoproducts, corresponding to ~5.0 x 10^3/cell/J/m^2. Therefore, at 40 J/m^2, the fluence used for the remainder of this study, the TA* content of a cell upon initial irradiation was ~2.0 x 10^4 lesions.

Repair of TA* photoproducts in normal human fibroblasts (GM38). Following the same procedure as used to study the induction rate of TA* lesions in genomic DNA, post-UV incubated normal human fibroblasts were assessed for their ability to repair this minor photoproduct. These experiments are summarized in the top half of Table XIII. Basically both purification steps, dialysis and DEAE-cellulose chromatography, were required to reduce thymidine to levels such that the d-T{p}A peak, arising after nuclease P1 digestion of the trinucleotides and eluting at fraction 48, was not contaminated by the tailing of the thymidine peak. Even with this extensive purification, and thus very little free thymidine contaminating the trinucleotides preparation, HPLC analysis of the subsequent nuclease P1 digestion exhibited only a small portion of radioactivity at the retention time of d-T{p}A (i.e. 2.9% in UV-irradiated, unrepaired DNA.) This was not because of a large thymidine peak as was often the case (see, for example, Figure 5, page 51), but because the other classes of lesions occurred more frequently and therefore generated the major radioactive peaks (see Figure 25).

The percentage of total radiolabel migrating at the position of the TA* lesion quickly diminished with post-UV incubation. By 12 hr post UV, a d-T{p}A peak was no longer distinguishable from the small amount of radioactivity eluting at this position when sham-irradiated were assayed (data not shown).

The absolute numbers of all three classes of lesions [cyclobutane dimers, (6-4) photoproducts, and TA^* lesions] have been calculated (in a similar manner as was done for the induction rates illustrated in Figure 24) and are presented in Figure 26. [Background radioactivity co-migrating at the positions of the various lesion-containing dinucleotides, as ascertained by analysis of sham-irradiated controls, has been subtracted from these values (data not shown).] Clearly, the DEAE-cellulose enrichment step did not cause a loss of lesion-containing trinucleotides, as the repair kinetics for cyclobutane dimers and (6-4) photoproducts were, within experimental error, identical to those presented in Figures 7 and 8 (see pages 53 and 54). Furthermore, it is evident that TA^* lesions were excised very rapidly, with kinetics not unlike those for (6-4) photoproducts, being essentially absent from the genome by 6 hr post UV.

Repair of TA^* photoproducts in fibroblasts representing the various XP complementation groups. The repair of TA^* dimers was ascertained in the eight different XP complementation groups (A through G and variant) using the same methodology as described above for normal fibroblasts. Only the results for XP variants are presented in any detail (see the bottom half of Table XIII) and are clearly insignificantly different from the data for normal cells.

To summarize the evaluations for the remaining seven XP complementation groups, the absolute number of TA^* dimers remaining in their genomes, at the different post-UV incubation times investigated, were computed. These are presented in Figure 27 along with the repair kinetics for XP variants and the normal cell stain (GM38). It is apparent that, like cyclobutane dimers and (6-4) photoproducts, the various XP complementation groups showed an assortment of abilities to repair the novel TA^* lesion. Groups A, F, and G all failed, completely, to remove this photoproduct. XP group B cells eliminated only a small percentage (~10%), whereas XP variants were entirely normal, having removed all of these dimers within 6 to 12 hr post UV. Group E cells also repaired all of the TA^* lesions induced in their genome by the irradiation conditions employed here, but at a reduced rate. Instead of having eliminated these adducts quickly, restoration occurred more slowly, being completed by 24 hr after treatment. Both XP group C and D eliminated ~40% of TA^* photoproducts over the 24 hr post-UV incubation period studied, but again the kinetics of this process differed markedly. Group D cells repaired this limited number of TA^* lesions with seemingly normal kinetics, whereas XP-C cells displayed a constant removal rate.

TA* photoproducts and (6-4) photoproducts are repaired with very similar kinetics. Along with the TA* data, Figure 27 also includes the repair of (6-4) photoproducts determined in this same study. It is immediately obvious from this direct comparison that (6-4) photoproducts and TA* lesions were repaired in each cell type with the same proficiency. That is, a total defect in (6-4) photoproduct repair paralleled a total defect in the removal TA* dimers; a reduced ability to repair the former lesions corresponded to the identical decline in TA* removal.

DISCUSSION

The minor photoproduct, the TA* adduct, was first described in 1983 (Bose *et al.*, 1983), but there has since been little advance in the understanding of its potential contribution to UV-induced cytotoxicity and mutagenesis. To assess the ability of various human fibroblast strains to repair this damage, a detection scheme was first necessary. This was accomplished in a manner similar to that done previously for (6-4) photoproducts and cyclobutane dimers (see Chapter 2). [^{3}H] thymidine-labelled d(AT)$_n$ was irradiated with a superlethal fluence (10 kJ/m^2) of 254-nm light. Enzymatic digestion produces lesion-containing trinucleotides, as well as mononucleosides. The trinucleotides were concentrated by dialysis plus DEAE-cellulose chromatography, and subsequently analyzed by HPLC chromatography. The two thymidine-elimination steps (dialysis and DEAE-cellulose chromatography) were essential to accurate quantitation of the TA* lesions present in any given preparation. This is because, with the HPLC column and gradient system employed, this latter species eluted shortly after thymidine. Even after dialysis, the amount of radioactivity present in thymidine was large enough that there was significant tailing of radiolabel in the region of interest. The second trinucleotide concentration step solved this problem (see Figure 25). Lesion-containing trinucleotides were unlikely lost during this purification procedure, as will be discussed later.

HPLC analysis of this concentrated sample revealed one major and novel peak (Figure 23A), and given that the only adjacent bases are thymine and adenine, was presumed to be the TA*-containing trinucleotide, d-ApT{p}A. In support of this deduction, nuclease P1 digestion of the same material altered the retention time of the major component, but not the percentage of total radioactivity associated with it (Figure 23B). This was expected because, since the predominant labelled lesion-containing trinucleotide was presumably d-ApT{p}A, and as thymidine carried the only label, loss of the 5' adenosine and phosphate by NP1/CAP digestion would not alter the percentage of total radioactivity associated with the TA* peak, d-T{p}A.

The chromatogram depicted in Figure 23B reveals a significant increase in thymidine as a result of nuclease P1 digestion. This would be predicted only if the initial preparation contained a trinucleotide with a 5' thymidine. Given the structure of the polymer, the only possibility would be an A-T lesion-containing trinucleotide, which has not yet been described. However, its existence may, in part, explain some of the minor peaks present in Figure 23A.

The data depicted in Figure 24 illustrates induction kinetics for cyclobutane dimers, (6-4) photoproducts, and TA* lesions, all quantitated from the same UV-irradiated DNA samples, following the two thymidine-elimination steps. The excellent agreement of both cyclobutane dimer and (6-4) photoproduct induction with that presented in Chapter 2 and as reported by others (Paterson *et al.*, 1973; Cadet *et al.*, 1983; Mitchell and Clarkson, 1984; Liuzzi *et al.*, submitted to for publication), argues against loss of photolesions during trinucleotide purification.

This investigation into the rate of induction of the minor TA* lesions discloses that they were generated by 254-nm light at approximately one-third the rate of (6-4) photoproducts (see Figure 24), corresponding to ~6% of the cyclobutane dimer frequency. This is significantly higher than their predicted occurrence of 1% of the level of cyclobutane dimers (Bose and Davies, 1984). Given the biological consequences of the cytotoxic (6-4) photoproduct (Cleaver *et al.*, 1988), although produced at only ~18% of the frequency of cyclobutane dimers, the TA* photoproduct cannot be considered irrelevant. Of course, at this time, the impact of the TA* lesion on the different cellular processes is unknown, but plans are underway to address this question (see Chapter 9).

Like (6-4) photoproducts, TA* lesions were eliminated from the genome very rapidly. Removal was complete by between 6 and 12 hr following 40 J/m^{2}. The parallel to (6-4) photoproduct removal is even more astonishing upon exploring TA* repair in the various XP complementation groups (see Figure 27). Without exception, TA* repair reflected the particular cell strain's ability to remove (6-4) photoproducts. For example, XP groups A, F, and G, which were not capable of (6-4) photoproduct repair, similarly did not eliminate TA* lesions. However, XP group D, which appeared to repair a subclass of (6-4) photoproducts, removed the same proportion of TA* lesions, and with seemingly the same normal kinetics. XP group C and E elicited the identical prolonged rate of TA* removal as they did for (6-4) photoproducts, and as was also the case for these latter lesions, XP-C removed only ~40% of the TA* adducts by 24 hr post UV, whereas XP-E eliminated them all. This result of parallel repair capabilities implies that these two minor lesions [(6-4) photoproducts and TA* lesions] are identified and removed by the same complex, which seems to not be the case for cyclobutane dimers (see page 39).

TA* adducts, like (6-4) photoproducts, appear to be impervious to the intradimer phosphodiesterase which modifies cyclobutane dimers. Evidence for this suggestion comes from analyzing excision fragments (data not shown). Cyclobutane dimers were found in two peaks upon enzymatic hydrolysis of UV-treated genomic DNA, but in four distinct peaks after enzymatic digestion of excision fragments (two for intact dimers and two for modified). However, (6-4) photoproducts were found in only one peak following hydrolysis of excision fragments. In addition, enzymatic digestion of UV-irradiated genomic DNA similarly produced one (6-4) photoproduct peak. The same was true for TA* lesions.

XP variants, as with cyclobutane dimer and (6-4) photoproduct repair, were indistinguishable from normal cells in their ability to remove TA* adducts. Therefore, the UV-sensitive phenotype of these cells cannot be explained by an inability to repair yet another minor photolesion.

Although very little is known about the cytotoxicity of the TA* lesion, it was repaired extremely rapidly in normal cells. This may imply that its elimination is essential to normal cellular function. Alternatively, it may only signify that because of common three-dimensional structure, they are repaired alongside (6-4) photoproducts. However, until more is known about these lesions, they should not be ignored when considering the cytotoxic and mutagenic damage caused by UV light. It will be interesting to pursue the question of the effect this novel lesion has on cellular processes such as replication and transcription (see Chapter 9).

cell strain	repair time (hr)	% dpm retained after dialysis (A)	% dpm retained on DEAE (B)	% dpm in TA* from HPLC analysis (C)	% total dpm in TA* (AxBxC) (X10^3)	TA* lesions remaining/cell (x10^4) *
GM38	0	1.96	12.7	2.9	7.1	23
GM38	3	1.83	11.3	1.6	3.4	11
GM38	6	1.26	14.3	1.4	2.5	8
GM38	12	1.04	10.2	1.2	1.3	4
GM38	24	0.89	10.5	1.0	0.9	3
GM38	sham-treated	1.01	9.0	1.0	0.9	3
CRL1166	0	1.92	10.7	3.4	6.9	22
CRL1166	3	1.75	10.2	1.9	3.4	11
CRL1166	6	1.29	11.7	1.5	2.2	7
CRL1166	12	1.18	9.5	0.5	0.6	2
CRL1166	24	1.13	10.4	0.5	0.6	2
CRL1166	sham-treated	1.07	10.5	0.5	0.6	2

TABLE XIII The removal of TA* lesions from genomic DNA in normal (GM38) and XP-variant (CRL1166) human fibroblasts.

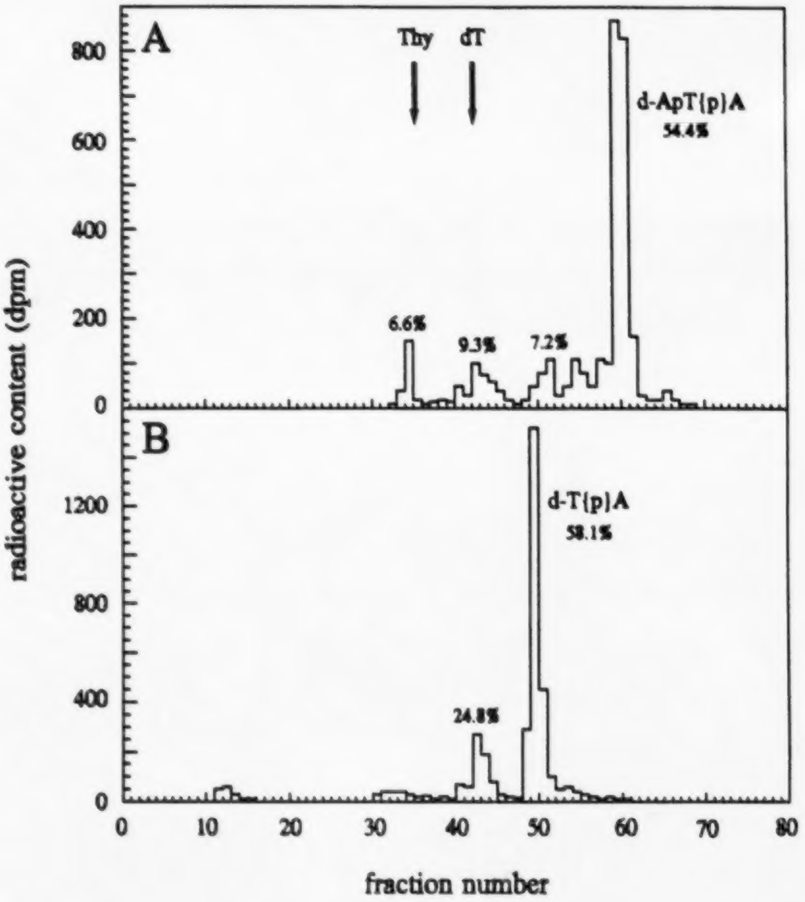


FIGURE 23 Reverse-phase HPLC chromatograms identifying the TA* photoproduct. **A.** TA*-containing trinucleotides after enzymatic digestion of UV-treated $d(AT)_n$ ([^{3}H] thymidine-labelled) and subsequent dialysis; **B.** d-T{p}A dinucleotides after NP1 digestion of the material in panel A.

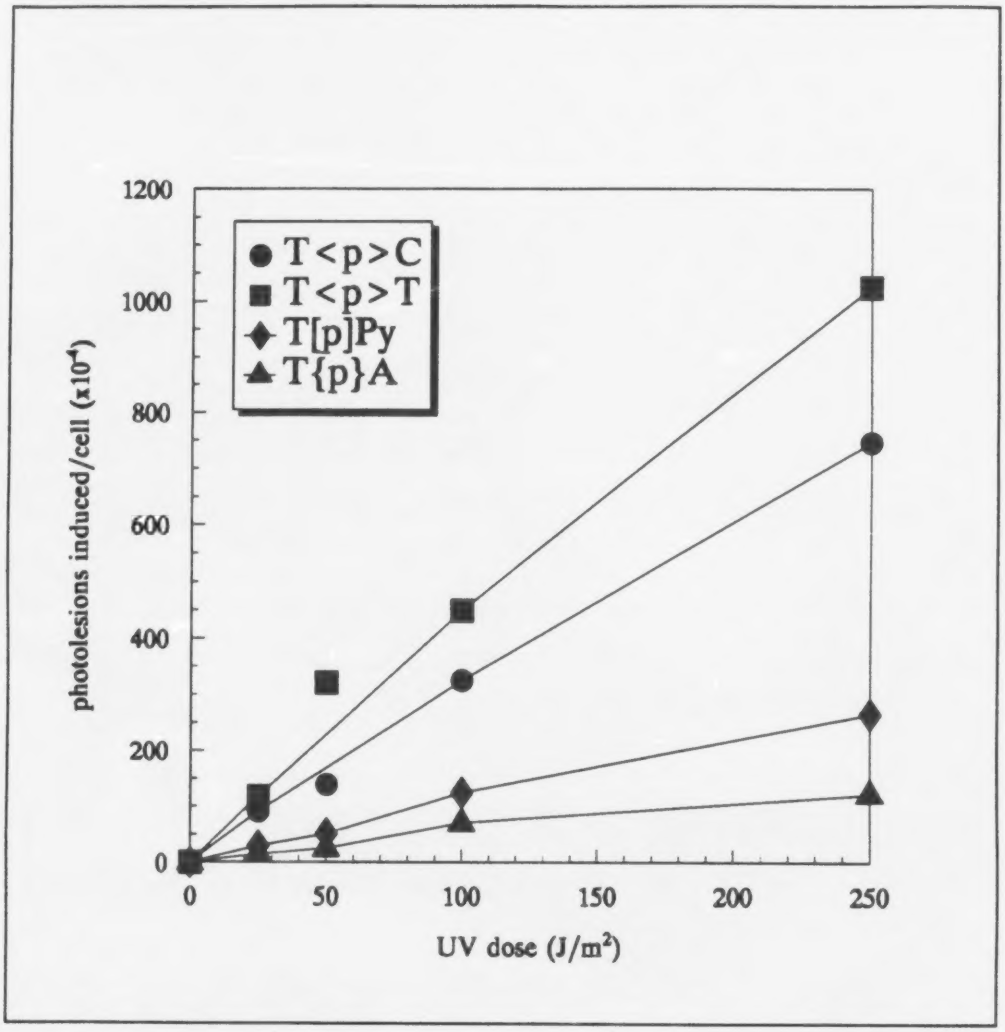


FIGURE 24 The rate of induction of cyclobutane dimers, (6-4) photoproducts and TA* lesions in genomic DNA as a function of 254 nm-light treatment.

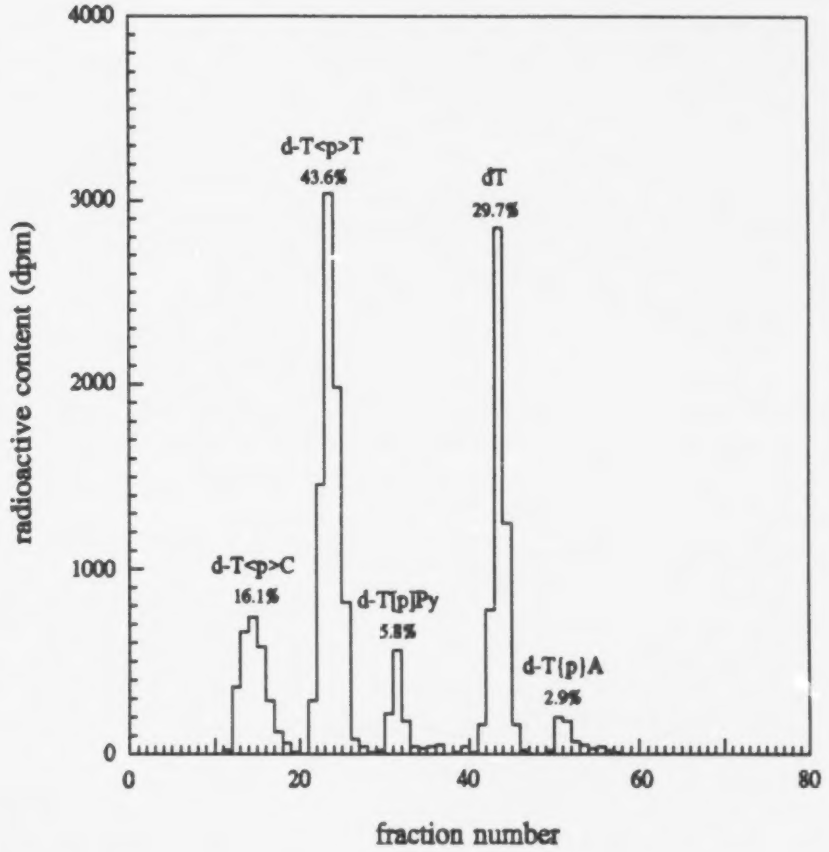


FIGURE 25 Reverse-phase HPLC chromatogram of nuclease P1-digested lesion-containing trinucleotides isolated from the genomic DNA of UV (40 J/m^2; 254-nm light) irradiated normal human fibroblasts (GM38). The small thymidine peak at fraction 42 allows quantitation of the d-T{p}A peak at fraction 48.

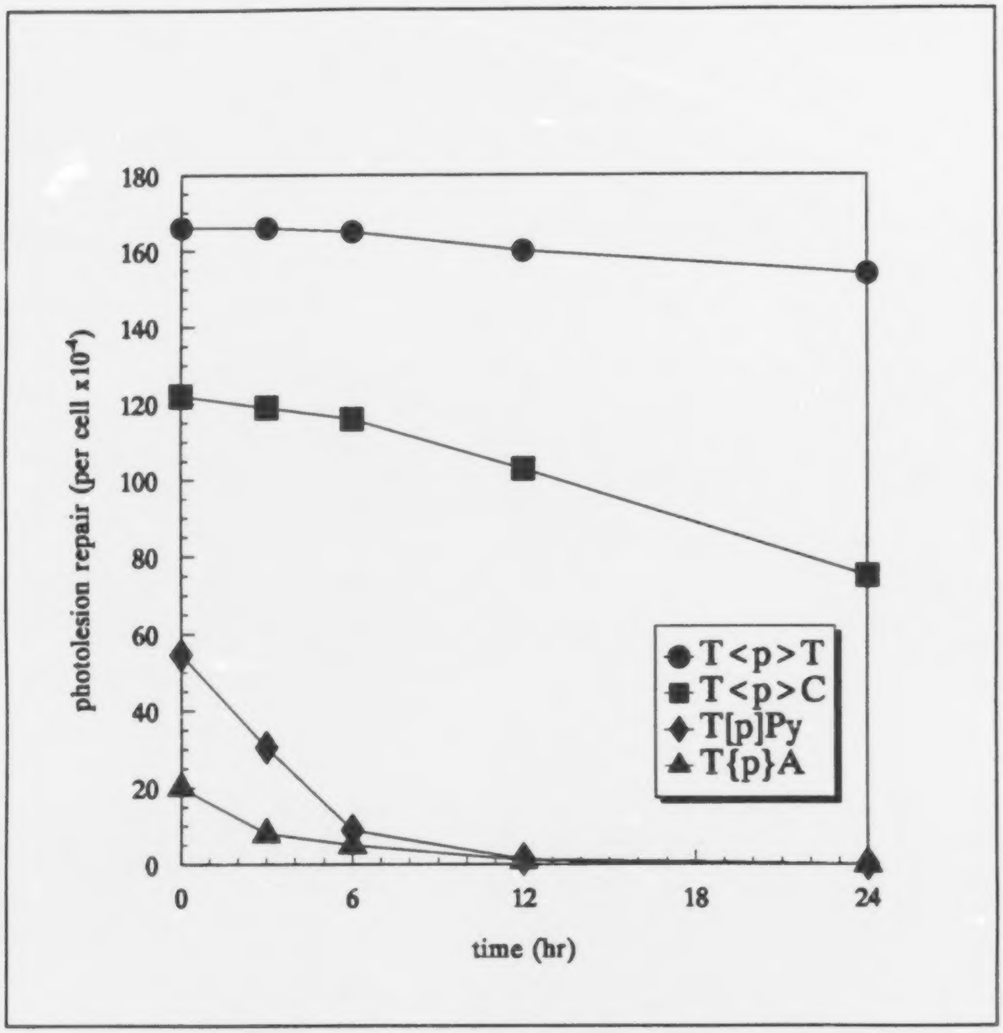


FIGURE 26 Repair of cyclobutane dimers, (6-4) photoproducts, and TA* lesions, following exposure of normal human fibroblasts to 40 J/m^2 of 254-nm light.

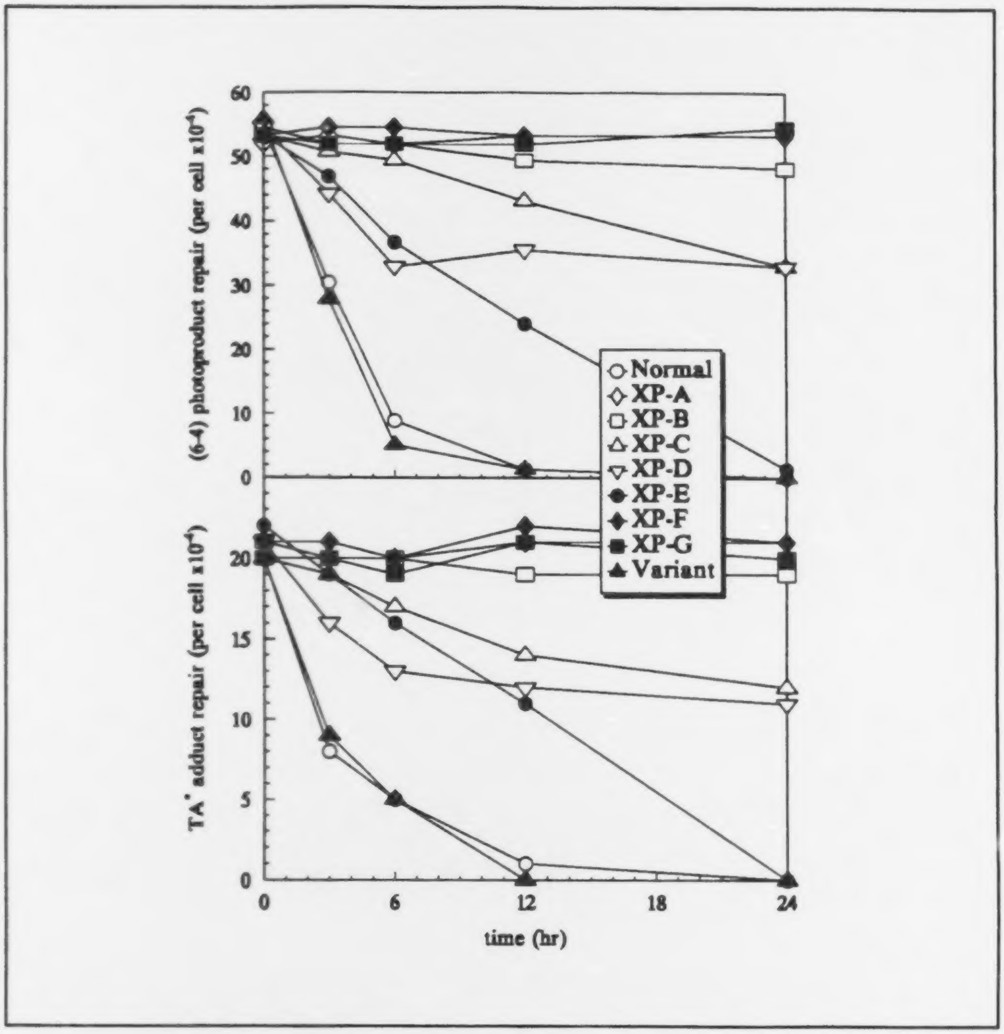


FIGURE 27 Repair of (6-4) photoproducts and TA* lesions in normal and various XP human fibroblasts following exposure to 40 J/m^2 of 254-nm light.

CHAPTER SEVEN - *ESCHERICHIA COLI* ALSO HAS INTRADIMER PHOSPHODIESTERASE ACTIVITY

INTRODUCTION

The repair of ultraviolet-induced DNA damage in *Escherichia coli*, by means of nucleotide excision repair, has been studied extensively [for reviews see Walker (1985); Rubin (1988); Grossman and Yeung (1990); van Houten (1990)]. It appears that we now understand almost every inherent interaction between each member of the recognition/incision complex (the UvrABC exonuclease) and the damaged substrate, as well as the ensuing single-stranded gap filling by DNA polymerase I. In recent years it has been proposed that *E. coli*, like mammalian cells, does not depend on the random removal of, at least, cyclobutane dimers to resume the vital cellular process of transcription. Instead repair enzymes appear to be shuttled, in some unknown way, to transcriptionally active sequences such that these are preferentially repaired (Mellon & Hanawalt, 1989).

To extend the ongoing studies in our laboratory on the role of an intradimer phosphodiesterase in the repair of UV-induced DNA damage in human cells, the existence of a parallel activity in *E. coli* was investigated. The validity of studying this prokaryote as a means of understanding the function of IDP in human cells is warranted by the parallels between *E. coli* and human nucleotide excision repair. The *E. coli* UvrABC exonuclease is able to complement the defective XP repair synthesis in a cell-free system (Hansson *et al.*, 1990), though this does not appear to be the case *in vivo*, presumably because of the inability of the UvrABC complex to attain access to chromatin (Zwetsloot *et al.*, 1986). Furthermore, although functional competition occurs between human nucleotide excision repair and *E. coli* photolyase and UvrABC nuclease, illustrating that human and *E. coli* repair enzyme subunits cannot substitute for one another within a repair complex, this demonstrates the overlap in substrate specificity and range between these two species (Ullah and Sancar, 1990). Moreover, as the genetics of this prokaryote are well documented and easily manipulated, this organism would serve as an ideal candidate to unravel the role of intradimer phosphodiester cleavage during post-UV repair, if such an activity did exist. This has turned out to be the case. IDP activity can be found in a crude protein extract from wild-type *E. coli*. In addition, excision fragments derived from post-UV incubated wild-type cells contain both cyclobutane dimers with an intact intradimer phosphodiester bond and with a severed interpyrimidine phosphodiester linkage. These observations have stimulated the further study of several previously identified *E. coli* mutants which display abnormalities in their response to UV irradiation.

The *E. coli* mutants which have been studied in detail, regarding their ability to sever the interpyrimidine phosphodiester bond of a cyclobutane dimer, include *uvrA*, *recA*, *recBC*, *lexA*, and *umuC*. The cellular functions of these genes can be summarized as follows. The gene products of *lexA* and *recA* regulate expression of the SOS genes. Many of the genes (the *din* genes) involved in the repair or management of UV-induced DNA damage belong to this SOS network (Kenyon and Walker, 1980). Their products are induced or upregulated in response to DNA damage. Lex A acts as a direct repressor molecule, binding to the promoter of each gene of this circuit. RecA*, upon induction, induces LexA to autocleave, resulting in the upregulation of the associated *din* genes (Witkin, 1976; Little and Mount, 1982). In addition, the nonregulatory action of RecA is required for recombinational bypass (Livneh and Lehmann, 1982) and recovery from inhibition of DNA synthesis following UV irradiation, a process independent of excision (*uvrA*), recombinational bypass (*recB*), and error prone DNA synthesis (*umuC*) (Khidhir *et al.*, 1985). UvrA protein (its cognate gene being a member of the SOS regulatory circuit) is an integral part of the UvrABC exonuclease and appears to be responsible for shuttling UvrB to the site of damage (Grossman and Yeung, 1990). UmuC is, in conjunction with UmuD, responsible for UV-induced mutagenesis. Its expression is also regulated by LexA and RecA, and functions by allowing a stalled replication polymerase to bypass a lesion with reduced fidelity (Bridges and Woodgate, 1985). The RecBC protein complex in an essential part of Chi-stimulated homologous recombination in *E. coli* (Stahl and Stahl, 1977).

It was anticipated that analyzing the expression of intradimer phosphodiesterase activity in these various mutants would reveal its association with the SOS regulatory circuit, the UvrABC complex, UmuCD-induced replication bypass, and recombination. No mutant studied to date exhibits a total deficiency of IDP activity. However, excision-deficient mutants (i.e., *uvrA*) accumulate backbone-cleaved cyclobutane dimer sites in their genomic DNA, implying that modification is not simply a post-excision event. Although we have proposed that modification of cyclobutane dimers somehow alleviates the block to progressing DNA and/or RNA polymerases, the IDP gene does not appear to be associated with the UmuCD pathway. It, therefore, may not function in replication bypass, at least through this mechanism.

EXPERIMENTAL PROCEDURES

For more detailed experimental procedures, see Appendix A, page 254.

Cell strains. The *E. coli* strains investigated included the following: **AB1157** [xyl-5, mtl-1, galK2, λ-rac$^-$, rpsL31, kdgk51, Δ(gpt-proA)62, lacY1, tsx-33, supE44, thi-1, leuB6, hisG4, mgl-51, arg-3, rfbD1, ara-14, thr-1], **TK603** (AB1157 arg$^+$, ilv^{ts}, uvrA6), **TK610** (AB1157 arg$^+$, ilv^{ts}, uvrA6, umuC36), **GW3198** (AB1157 umuC36), **GW2727** [AB1157 lexA3(Ind$^-$), malE::Tn10], **DL184** (AB1157 recB21, recC22), **JC9937** [thi-1, leu-6, proA1, his-4, argE3, mtl-1, lacY1, galK2, ara14, xyl-5, srlD, str31 (SmR), tsx-33], **SMR374** [JC9937 Δ(srlR-recA)306::Tn10]. The first five strains were gifts from Dr. Graham Walker, and the last three were supplied by Dr. Susan Rosenberg.

Use of excision fragments to investigate cyclobutane dimer modification.

Culture conditions and radioactive labelling of cellular DNA. The following were used in the experiments outlined below: M9CA (pH 7.4) medium consisting of 1.3% $Na_2HPO_4 \cdot H_2O$, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 2 mM $MgSO_4$, 0.2% glucose, 100 µM $CaCl_2$, and 0.2% casamino acids (Difco Laboratories, Detroit, MI); and NT buffer containing 0.1 M NaCl and 10 mM Tris-HCl (pH 7.4). Except where indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO)

A single bacterial colony was inoculated into 10 ml of M9CA supplemented with 1 µg/ml thiamine, and shaken overnight at 37°C. Five ml of the overnight culture (containing ~ 3×10^9 cells) were added to 200 ml of fresh M9CA + thiamine containing, in addition, 5 µCi/ml [^{3}H]-thymidine (58 mCi/ml; NEN Canada, Montreal, PQ) and 200 µg/ml 2-deoxyadenosine. The presence of 2-deoxyadenosine in the medium allowed the uptake and incorporation of thymidine into DNA in a nonthymidine-requiring cell. These cultures were incubated in 1-*l* flasks at 37°C for 2 hr, to re-establish logarithmic growth, and subsequently allow approximately 2 cell doublings (final concentration, ~6×10^7 cells/ml). Cells were then collected by centrifugation (6000*g* for 10 min at 4°C) washed once with ice-cold NT buffer, and resuspended in 0.5 volumes of the same buffer.

UV irradiation and subsequent incubation of bacterial cultures. A 20-ml aliquot of resuspended cells was placed in a 150-mm plastic tissue culture plate (GIBCO/BRL Inc., Burlington, ON) and irradiated, with mixing, with a total fluence of 40 J/m^2 of 254-nm light. (For lamp source and its specifications, see Chapter 2, page 15, 258). These UV-treated cells were then either placed immediately on ice (no repair), or added directly to 200 ml of prewarmed (37°C) nonradioactive thiamine-containing M9CA. Cultures were then incubated in the dark (to inhibit photoreactivation) at 37°C, with shaking, for various lengths of time (5, 15, 30, 45, 60, 90, and 120 min). A 20-ml aliquot of cells was also sham-irradiated and served as an unirradiated control. These cells were also added to 200 ml of M9CA and incubated for an additional 120 min. In experiments where the only goal was to ascertain whether or not modified dimers were present in excision fragments, the only post-UV incubation time studied was 2 hr.

Collection of excision fragments from post-UV incubated cultures. After post-UV incubation, cells were collected by centrifugation, washed twice with ice-cold NT buffer, and resuspended in 500 µl of 10 mM Tris-HCl (pH 7.5). To collect excision fragments, equal volume of 10% trichloroacetic acid was added to lyse the cells and release the small UV lesion-containing oligonucleotides. This mixture was stored on ice for 15 min before separating the TCA-soluble and insoluble fractions by centrifugation. The percentage of total radiolabel representing each fraction was determined, and the TCA-soluble material was saved for further analysis. This latter fraction was diethyl ether extracted as described in Chapter 2 (page 16). Because of the high concentration of unincorporated radioactively-labelled thymidine in the TCA-soluble fraction, the lesion-containing oligonucleotides were enriched by overnight dialysis, as described previously (Chapter 2, page 15).

Measurement of lesions present in excision fragments from post-UV incubated cells, and assessment of the state of the intradimer phosphodiester bond. Excision fragments were analyzed using the same three-stage enzymatic digestion/HPLC protocol described in Chapter 2 (page 16).

Assessment of UV lesions remaining in cellular DNA following post-UV incubation.

Isolation of genomic DNA from post-UV incubated cells. Growth conditions, radioactive labelling of cellular DNA, irradiation, and subsequent post-UV incubation were identical to that described above for excision fragments. Post-UV incubated cells were then resuspended in 2 ml of lysis buffer containing 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5% glycerol (v/v), and 300 µg/ml lysozyme, and incubated on ice for 1 hr. Cell lysis was completed by the addition of 0.05% Nonident-40 (v/v). Cell debris was removed by centrifugation (10,000*g* for 30 min at 4°C). The supernatant was phenol/chloroform-extracted, and the DNA was precipitated overnight at -20°C in 70% ethanol. The DNA pellet was collected by centrifugation (15,000*g* for 15 min at 4°C), taken to dryness, and resuspended in ddH_2O.

Analysis of the lesions present in genomic DNA isolated from post-UV incubated cells. Specific activity of the labelled DNA was ascertained by determining the radioactive content of a small aliquot in a liquid scintillation system. DNA containing ~2.5×10^7 dpm was digested with staphylococcal nuclease, DNase I, snake venom phosphodiesterase, and calf alkaline phosphatase as described in Chapter 2 (see page 15, 263, 274). The reaction products were then dialysed overnight (12 hr; see page 15) to concentrate the lesion-containing trinucleotides. After determining the percentage of radioactivity retained in the dialysis membrane, this material was subjected to the enzymatic hydrolysis/HPLC methodology described in Chapter 2 (page 16).

RESULTS

UV-photoproduct repair in wild-type *E. coli*. As a preamble to the study of cyclobutane dimer modification in *E. coli*, it was first necessary to ascertain whether or not this mechanism existed in this prokaryote. During this process, a detailed analysis of the repair kinetics for both cyclobutane dimers and (6-4) photoproducts was carried out using the enzymatic digestion / HPLC assay described in Chapter 2 (page 16).

During nucleotide excision repair in *E. coli*, the UV adducts which are removed from the DNA, accumulate in the cell as small lesion-containing oligonucleotides (La Belle and Linn, 1982). As is the case for mammalian cells (see preceding chapters), these excision fragments can be purified from the bulk genomic DNA by their characteristic solubility in trichloroacetic acid. Although the percentage of radiolabel recovered from *E. coli* as TCA-soluble material was very high (~25 - 30%; see Table XIV), the majority of this material proved to be unincorporated, unphosphorylated thymidine (data not shown). To enrich for the lesion-containing excision fragments, this material was subjected to dialysis (described in Chapter 2; page 15) prior to further analysis. This procedure reduced the radioactive content of the oligonucleotide-containing fraction to less than 5% of the starting material (see Table XIV).

The photolesions contained within the excision fragments derived from post-UV (40 J/m^2) incubated [^{3}H]-thymidine labelled cells were quantitated. A sample HPLC chromatogram is depicted in Figure 28 for 2 hr post-UV incubation, and data for all repair times, as well as for sham-irradiated cells, are summarized in Table XIV. As is readily evident from these data, signs of nucleotide excision repair events were already evident by 5 min following UV exposure, with 1.4% of the total radioactivity represented by the various photodimer peaks. The percentage of radioactivity associated with both T^C and T^T cyclobutane dimers continued to rise with increasing repair time up to the longest (2 hr) post-UV time studied. The T^C peaks (d-T<p>C and dT<>dC) increased from 0.2% to 2.9% of the total radiolabel during this time. The T^T peaks (d-T<p>T and dT<>dT) increased from 0.6% to 4.8%. The same was not true of the radioactivity associated with (6-4) photoproducts, however. By 15 min post UV, the percentage of total radioactivity associated with these lesions had increased from 0.6% to 0.9%, but there was no further increase with extended post-UV incubation time.

The absolute number of excised lesions per cell has been calculated based on the assumptions presented in the legend of Table XIV, and are displayed in the same table. The same results are presented for immediate visualization in Figure 29. Clearly, (6-4) photoproducts were a predominate lesion appearing in excision fragments immediately following UV exposure. After only 5 min post-UV (40 J/m^2) incubation, there had been 110 of these lesions excised per cell, as compared to 37 for T^C dimers and 55 for T^T dimers. By 30 min following irradiation, there was no further accumulation of (6-4) photoproducts in excision fragments, implying that the repair of this class of photolesion had been completed (see Figure 29). In contrast, the number of excised cyclobutane dimers continued to climb (to 518 T^C and 428 T^T per cell) up to the longest post-UV incubation time assayed (2 hr).

Figure 28 clearly illustrates that modified cyclobutane dimers were present in excision fragments isolated from post-UV incubated *E. coli* cells. The percentage of cyclobutane dimers which have a either severed intradimer phosphodiester bond, or have been left unmodified, has been calculated for the various repair times, and is presented in Table XV. Although the proportion of dimers found in excision fragments which had their interpyrimidine phosphodiester bond cleaved was moderately high at 5 min following irradiation (48%), there was a general trend suggesting that the fraction of lesions which were modified increased with extended post-UV repair period (see also Figure 30). At 15 min post UV, only 26% of the cyclobutane dimers had a cleaved interpyrimidine phosphodiester bond, but this percentage increased constantly, such that by 2 hr post UV, the majority of these photoproducts (57%) were modified. Moreover, at 2 hr post UV, T^T dimer were more likely to be modified than T^C dimers, with 67 and 39% modified, respectively.

Photoproduct removal in various *E. coli* mutants. To assess the ability of several *E. coli* mutant strains to modify cyclobutane dimers, excision fragments were collected from post-UV (40 J/m^2) incubated (0 or 2 hr) cells (as well as sham-irradiated cells; data not shown), and analyzed by the enzymatic hydrolysis/HPLC methodology. Results from this study are presented in Table XVI. The absolute numbers of lesions excised per cell have again been calculated, based on the same assumptions presented in Table XIV, and are also displayed in Table XVI. Although this study gave no information on the kinetics of photoproduct removal, it was quite clear that cells of the second wild-type strain (JC9937), as well as *umuC*, *lexA*, and *recBC* cells, had excised photoproducts, by 2 hr following 40 J/m^2, to an extent indistinguishable from that established by AB1157 wild-type cells. After subtracting background, these values for *umuC*, *lexA*, and *recBC*, respectively, were 494, 504, and 423 T^C lesions per cell, 511, 437, and 441 T^T dimers per cell, and 142, 137, and 152 (6-4) photoproducts per cell. The *recA* strain, however, displayed a significant cyclobutane dimer repair deficiency, having removed, for example, only about 65% (or 311 per cell) of the T^C dimers repaired by the other cell strains in the same 2 hr post-UV period. In contrast, the number of (6-4) photoproducts found in excision fragments (141 per cell when background is subtracted) was essentially the same as for all other strains.

Cyclobutane dimers with a severed intradimer phosphodiester bond were detected in excision fragments derived from all four of these mutant strains. The distribution of the two configurations of cyclobutane dimers (i.e. modified and intact) has been tabulated (see Table XVII) for these mutants, as well as for the two wild-type strains. The excision fragments (2 hr post UV) derived from both wild-type *E. coli* strains (AB1157 and JC9937), contained 57% and 47%, respectively, of their cyclobutane dimers in a modified form. Correspondingly treated strains, harboring a mutation in either *umuC* or *lexA*, also displayed a similar percentage of cyclobutane dimers which had their intradimer phosphodiester bond cleaved (45 and 48%, respectively). The content of modified dimers was slightly lower (38%) in excision fragments isolated from the *recBC* strain, and reduced even further in the *recA* mutant (25%). However, none of these strains exhibited a complete inability to cleave the intradimer phosphodiester bond of cyclobutane dimers found in excision fragments.

Accumulation of modified cyclobutane dimers in the DNA of excision-deficient mutants. The types of lesions remaining in the DNA of two post-UV incubated *E. coli* excision-deficient strains, were ascertained using the enzymatic hydrolysis / dialysis / HPLC methodology. Both of these strains harbored the *uvrA* mutation. One strain (TK610) contained, in addition, a mutation in the *umuC* gene. Results of this study are presented in Table XVIII. Even in the earliest sample, there was already evidence of modified cyclobutane dimers in the genome. Thus, this repair time has been designated 5 min post UV, as this is approximately the length of time taken to lyse the cells following irradiation. It was impossible, under the irradiation/cell collection conditions used, to achieve an authentic 0 hr repair time point. However, results from sham-irradiated controls indicated that the radioactive peaks presumed to be associated with these modified cyclobutane dimers represented authentic UV-induced damage, and were not radioactively-labelled species, unrelated to UV-irradiation, which were retained at these same positions on the HPLC chromatogram. Furthermore, measurements of the total cyclobutane dimer content, ascertained by formic acid hydrolysis, of the TCA-soluble material isolated from irradiated cells (harvested immediately following UV-irradiation, i.e., 5 min repair), were consistent with the modified dimer peaks corresponding to authentic cyclobutane dimers (data not shown). Moreover, photochemical reversal of material isolated from the dT<>dC and dT<>dT peaks, confirmed that these species were, in fact, modified cyclobutane dimers (data not shown).

Because of the small percentage of total radiolabel which was associated with the various lesion peaks, a large amount of radioactivity ($\sim 10^7$ dpm) was analyzed. Thus, peaks comprising only 0.1% of the total radioactivity still contained 10,000 dpm, and therefore could be assessed with certainty. In material isolated from the *uvrA* strain, almost immediately following irradiation with 40 J/m^2, there was 0.04% and 0.13% of the total radioactivity in the analyzed fraction which was associated with the two types of modified dimers (i.e., dT<>dC and dT<>dT). This did not significantly change after 2 hr of post-UV incubation (0.04% and 0.1%, respectively), nor did the addition of the *umuC* mutation make any difference in this value (0.04% and 0.12% at 5 min, respectively). The absolute numbers of the various photoproducts remaining in the genome of the two repair-deficient strains have been calculated, based on the assumption presented in Table XIV, and are displayed in Table XVIX. Under the irradiation conditions employed (40 J/m^2), approximately 175 (6-4) photoproducts, 570 T^C dimers, and 685 T^T dimers were present in the genomic DNA of each cell after 5 min post UV. These values, as expected, did not diminish with repair time in these excision-deficient strains. The percentage of cyclobutane dimers which had a severed interpyrimidine phosphodiester bond have also been determined (see Table XX). Of the cyclobutane dimers initially introduced, both strains studied had roughly 14% of their cyclobutane dimers modified at both repair times.

The method employed to enrich a DNA sample for lesion-containing trinucleotides (i.e., enzymatic hydrolysis followed by dialysis) likely resulted in an underestimation of the total number of cyclobutane dimers with an intradimer phosphodiester cleavage residing in genomic DNA. As a modified cyclobutane dimer, contained in a trinucleotide and generated by enzymatic digestion, would retain only one phosphate group, there would be loss of this species during the subsequent dialysis step (~60% in the 12 hr dialysis period employed in this study; see Chapter 2, Figure 2, page 48). Therefore, modified dimers may represent as many as 30% of the cyclobutane dimers remaining in the genomic DNA of these repair-deficient mutants.

DISCUSSION

Escherichia coli has served as a model system for the study of many cellular processes. It has been well-characterized genetically due to its relative simplicity and ease of genetic manipulation. We have initiated studies to investigate the existence of an activity which cleaves the intradimer phosphodiester bond of a cyclobutane dimer in *E. coli*. The existence in human cells of such an activity has previously been described by our laboratory (Paterson *et al.*, 1984; Weinfeld *et al.*, 1986; Paterson *et al.*, 1987), and the responsible protein has now been purified to almost complete homogeneity (Liuzzi *et al.*, manuscript in preparation). Although cloning of the cognate gene has been initiated, typical difficulties associated with cloning genes in human cells have been encountered (Lehmann, 1985; Schultz *et al.*, 1985; Gebara *et al.*, 1987; Mayne *et al.*, 1988), and progress has therefore been slow. As *E. coli* is a much simpler system to work with, it was reasoned that an understanding of the functionality of IDP could be hastened by analyzing the parallel activity in this organism. This, of course, was dependent on such an activity occurring in prokaryotic cells and, in itself, was an interesting question as an earlier assumption had been made that IDP was specific to mammalian cells (Paterson *et al.*, 1987).

The study of excision fragments isolated from post-UV (40 J/m^2) incubated wild-type *E. coli* has clearly demonstrated that, like human and CHO cells, a large proportion of cyclobutane dimers contained within the small oligonucleotides arising during nucleotide excision repair had a severed intradimer phosphodiester bond (see Figure 28). This observation, then, stimulated further exploration of UV photoproduct repair in wild-type *E. coli* and assessment of several *E. coli* mutant strains with regards to their ability to sever the interpyrimidine phosphodiester bond of cyclobutane dimers.

Various repair times were assessed during the course of characterizing excision fragments derived from wild-type *E. coli*. This was done, initially, to determine if the proportion of modified cyclobutane dimers contained in excision fragments changed as a function of post-UV incubation period. However, this study also revealed other similarities between prokaryotic and mammalian DNA repair. Although *E. coli* removed all photoproducts in a much shorter repair time than did human or CHO cells, in all cases, the percentage of total (6-4) photoproducts removed at early repair times was higher than the percentage of cyclobutane dimers (see Figure 29 and Figure 8, Chapter 2). In the case of *E. coli*, these lesions had been almost entirely eliminated within 5 min following UV (40 J/m^2) treatment. For human cells, this occurred by about 6 hr post UV. In both instances, this class of lesions had been eradicated within approximately one-quarter of the time required for cell division.

Parallels can also be drawn regarding cyclobutane dimer repair in these different species. Modified dimers were present in the excision fragments of all three species (*E. coli*, Chinese hamster and human). Furthermore, in all instances, at very early repair times, the number of cyclobutane dimers which had been eliminated from the genome was outnumbered by (6-4) photoproducts, even though the latter lesions were induced at only ~15% of the frequency of cyclobutane dimers. And finally, although induced at a lower rate, T^C lesions were repaired more rapidly [they outnumbered T^T lesions found in excision fragments 1.2-fold by 2 hr post UV in *E. coli* and by 24 hr in human cells (see Figure 29 and Figure 7, Chapter 2).] This suggests that these mechanisms [i.e. intradimer cleavage, selectively rapid (6-4) photoproduct removal, and seemingly more efficient recognition of a T^C lesion than of a T^T lesion] have been conserved through evolution. Preferential repair of transcriptionally active genes also appears to fall into this category (Mellon and Hanawalt, 1989).

As is illustrated in Figure 30, there was a trend towards a higher proportion of cyclobutane dimers having a severed intradimer phosphodiester bond with increased repair time. The exception to this was at the earliest repair time (i.e. 5 min), but given the small number of lesions excised at this time, accurate values cannot be expected. The overall increase in excised modified dimers with post-UV incubation period has different interpretations. It could be assumed that modification is a post-excision event and therefore the proportion would increase with time. However, several lines of evidence argue against this being, at least, the total picture. First of all, excision-deficient mutants [specific XP complementation groups in humans (Paterson *et al.*, 1987) and *uvrA E. coli* cells (see below) accumulate these sites in the genomic DNA. Secondly, modified dimers have been observed transiently in transcriptionally active genes in normal human cells (M.V. Middlestadt, K. Dietrich, and M.C. Paterson, unpublished data). Thirdly, it has since been found that at least human liver IDP does not operate on double-stranded or single-stranded molecules of less than 25 base pairs or nucleotides (see Chapter 8). Nevertheless, in wild-type *E. coli* there was a slight decrease at longer repair times (see Figure 30) in the number of cyclobutane dimers, contained within excision fragments, which had an intact intradimer phosphodiester bond. This implies that there is some activity present in the *E. coli* cell which is active on the intradimer phosphodiester linkage between dimerized pyrimidines contained in short single-stranded oligomers.

Although dimer modification has been proposed to be a necessary pre-incision event in human cells by enabling repair complex recognition of cyclobutane dimers (Paterson *et al.*, 1987), the data presented here indicate that this is unlikely the case for *E. coli*. Although it has been found that the UvrABC exonuclease of *E. coli* has lower affinity for cyclobutane dimers than it does for other types of damage (Van Houten *et al.*, 1986; Grossman *et al.*, 1988; Lambert *et al.*, 1989), and therefore it could be argued that modification by intradimer phosphodiester cleavage might increase the chemical distortion, thus aiding recognition by the incision complex, the appearance of both modified and intact cyclobutane dimers in excision fragments argues against this premise. Furthermore, the analogous result in both human and CHO cells (see Chapters 2 and 3) indicates that IDP action is also not a critical pre-incision event in mammalian excision repair.

Our working hypothesis for IDP function proposes that modification of cyclobutane dimers is a cellular mechanism to cope with unrepaired damage by releasing helical stress, thereby allowing the replication and/or transcription machinery past these otherwise polymerase-blocking lesions. If modification decreases the distortion caused by the cyclobutyl bridge, this would be expected to result in less efficient recognition of these dimers by the incision complex. A predicted outcome, if this were the case, would be a greater proportion of repaired cyclobutane dimers at early post-UV incubation times having an intact phosphodiester linkage than at later times, as these would be more easily identified and therefore eliminated more effectively. The data on the distribution of the various excision fragment-containing lesions at succession post-UV incubation times in *E. coli* supports this prediction.

Having confirmed that severage of the intracyclobutane dimer phosphodiester linkage occurs in wild-type *E. coli*, the state of this bond was assessed in excision fragments or genomic DNA isolated from several *E. coli* mutants with the anticipation of uncovering an IDP-deficient mutant. As summarized above, one hypothesized function of IDP is to permit replication bypass. The process of UV-induced mutagenesis is accomplished by a misincorporation event at the replication fork across from a photoproduct and subsequent replication bypass. The UmuCD complex functions in allowing the replication machinery to pass the non-base pairing error. It thus rescues the stalled polymerase, in some yet unknown way, rather than being, itself responsible for the misincorporation event (Bridges and Woodgate, 1985). This renders the *umuC* and *umuD* loci as candidate IDP genes. Analysis of excision fragments derived from post-UV incubated *umuC* cells (see Tables XVI and XVII) clearly indicated that this gene is not responsible for intradimer phosphodiester cleavage, as the number of modified dimers contained in excision fragments derived from post-UV incubated *umuC* cells was indistinguishable from wild-type.

The genes whose protein products comprise the repair exonuclease, as well as several other loci, are upregulated during the SOS response. The two key players in the control of this regulatory circuit are the *lexA* and *recA* gene products. LexA acts as the repressor to every known gene in the SOS circuit, although most SOS genes are expressed at significant levels even in the absence of induction (Walker, 1985). RecA has many cellular functions, but its most central role in the SOS response is to induce autocleavage of LexA, thereby rendering it inactive. RecA is activated by single-stranded DNA or DNA degradation products, which are abundant following any event which triggers the SOS response, including exposure to UV radiation (Witkin, 1976; Little and Mount, 1982; Sedgwick, 1986). It was surmised that, by studying the ability of *lexA* and *recA* mutants to modify cyclobutane dimers, it would be possible to determine whether or not the IDP gene is a member of the SOS regulatory circuit.

Excision fragments collected from post-UV incubated (2 hr) *lexA* cells are indistinguishable from those isolated from wild-type cells, both with regard to the numbers of (6-4) photoproducts and cyclobutane dimers they contain (see Table XVI) and the proportion of dimers modified. In contrast, the *recA* mutant excised a significantly lower number of cyclobutane dimers. As well, a smaller proportion of the excised dimers were modified. The *lexA* mutant used in this study, *lexA*(Ind-), renders the SOS response noninducible. The excision of a wild-type number of photolesions by 2 hr post UV in this strain can be explained by the significant basal levels with which the *uvrA*, *uvrB*, and *uvrC* gene products are produced in non-induced cells. The wild-type distribution of intact versus modified dimers implies that the IDP gene is not a member of the SOS regulatory circuit, unless it is produced at a basal level high enough that further induction of the gene does not change the distribution of these two classes of cyclobutane dimers within excision fragments. The low level of both lesion excision and dimer modification in the *recA* mutant may simply reflect the deleterious effects of completely eliminating RecA function, as this gene product is involved in several cellular processes. The *recA* data, therefore, adds no addition insight into the relationship of the IDP gene and the SOS circuit. However, one may speculate that RecA is involved in IDP activity based on the following scenario. Following UV irradiation, DNA synthesis is inhibited. Inhibition appears to result solely from the DNA damage itself, and not due to an inducible function (for review see Walker, 1985). However, the resumption of DNA synthesis requires both an inducible factor, Irr (*i*nduced *r*eplisome *r*eactivator), as well as the nonregulatory action of RecA protein (Khidhir *et al.*, 1985).

Recovery from inhibition of DNA synthesis does not require *uvrA* (excision repair), *recB* (recombinational bypass), or *umuC* (error prone DNA synthesis) (Khidhir *et al.*, 1985). A favored premise for the combined action of RecA and Irr is in allowing translocation of the replicative polymerase past non-instructive lesions in an uncoupled reaction, with reinitiation upstream (Khidhir *et al.*, 1985). This would then account for postreplication gaps. The actual mechanism of action proposed for the Irr-RecA complex involves modification of the replisome (Khidhir *et al.*, 1985). It is well-documented that cyclobutane dimers are polymerase-blocking lesions (Caillet-Fauquet *et al.*, 1977; Villani *et al.*, 1978; Moore *et al.*, 1981; Vos and Rommelaere, 1982; Griffiths and Ling, 1989). If RecA were able to cleave the intradimer phosphodiester bond, and thereby allow translocation past the lesion, this would account for the direct role of RecA in recovery from inhibition of DNA synthesis. However, the presence of modified dimers in excision fragments derived from post-UV incubated *recA* cells indicates that this is not the case. Nonetheless, an as of yet untested possible IDP function, not inconsistent with the data presented here, is that the Irr factor and IDP are one and the same. Although there is as yet no evidence that IDP is inducible, cyclobutane dimer modification may well allow translocation of the DNA polymerase past this non-coding lesion.

There are several cellular processes that transiently generate single-strand breaks, such as the action of topoisomerases (Gedik and Collins, 1990) and recombinases. The best understood recombination enzymes in *E. coli* are RecA and RecBCD. RecA is required for recombinational bypass of pyrimidine dimers (Livneh and Lehmann, 1982). As documented above, RecA influences IDP action, but this role appears to be in conjunction with SOS control. The most current model for RecBCD involvement in homologous recombination has it loading onto the end of a double-stranded break and degrading the DNA until it reaches a Chi site (a specific octomer sequence), where it then becomes a helicase. The resultant single-stranded DNA is recombinogenic (Rosenberg and Hastings, 1991). However, RecBC also confers single-stranded endonuclease activity, and as protein can often have several seemingly unrelated functions (for e.g., RecA both acts to induce autocleavage of LexA, and participates directly in recombination), a *recBC* mutant was assayed for IDP activity. As illustrated in Tables XVI and XVII, excision fragments derived from post-UV incubated *recBC* cells contained modified dimers in a proportion similar to that seen in wild-type cells. The RecBC enzyme, therefore, unlikely has intradimer phosphodiesterase activity. Mutants with defects in other genes coding for known endonucleases or topoisomerases have not been tested as yet.

If modification of cyclobutane dimers occurs to enable DNA and/or RNA polymerases to navigate past these non-coding lesions, it is predicted that, following UV irradiation, a repair-deficient mutant will accumulate such sites in its genome. To test this prediction, genomic DNA was isolated from post-UV (40 J/m^2) incubated *uvrA* cells and analyzed for the types of lesions present. As indicated in Table XVIII, even at the most immediate time that DNA could be collected from these cells (~5 min), there was already evidence of cyclobutane dimer modification. Attempts to document a true zero time point proved unsuccessful, partially because of the procedures involved in isolating genomic DNA. However, formic acid hydrolysis and photochemical reversal of the presumed modified cyclobutane dimer-containing material, as well as results obtained for sham-irradiated cells, confirmed that this almost immediate cyclobutane dimer modification was authentic. This implies that cyclobutane dimer modification occurs extremely early in *E. coli* following UV-induced DNA damage, with as many as 30% of these lesions undergoing intradimer phosphodiester cleavage. Data from a later repair time (2 hr) indicated no real increase in the number of dimers with a severed intradimer phosphodiester bond with continued post-UV incubation. The absolute number of modified sites in the genome of a *uvrA* mutant has been compared to the number of these dimers appearing in excision fragments isolated from post-UV incubated wild-type *E. coli* (see Table XXI). Although the yield of cyclobutane dimers with a severed interpyrimidine phosphodiester linkage appearing in excision fragments isolated from wild-type cells that have been incubated for 5 min after UV treatment was approximately the same as the number of these sites present in the genome of the repair-deficient *uvrA* strain at the same post-UV repair time, the same was not true after 2 hr. At this later time, there were 2.5 times as many modified dimers excised in wild-type cells as could be found in the genomic sequences of *uvrA* cells. There are two ready explanations for this discrepancy. Dimer modification could occur post-excisionally, as well as pre-excisionally, as a mechanism to rid the cell of the dimerized nucleotides which would otherwise carry an internal charge. This would also explain the decreasing number of intact dimers contained within wild-type excision fragments at longer repair times. Alternatively, dimer modification could occur transiently.

In summary, severage of the intracyclobutane dimer phosphodiester linkage occurs in *E. coli* as well as in human and CHO cells. The presence of these sites in genomic DNA isolated from post-UV incubated *uvrA* cells, and the occurrence of both intact and modified dimers in excision fragments derived from post-UV incubated wild-type cells, suggests that this activity has a pre-excision function not associated with nucleotide excision repair per se, although there is evidence of some post-excision modification as well. Screening of a limited number of *E. coli* mutants, with phenotypes or cellular activities suggestive of IDP activity, has failed to uncover an IDP mutant. However, there are several UV-sensitive *E. coli* mutants, which have not been examined, which may still disclose the appropriate loss of IDP function. Alteratively, isolation of the IDP protein may more quickly reveal the function of this nuclease.

repair time (min)	% of total radiolabel in TCA-soluble fraction	% of TCA-soluble radiolabel retained after dialysis	% of total radiolabel analyzed	% Thy residing in UV lesions in analyzed fraction			UV lesions excised per cell		
				T^C	T^T	T[p]Py	T^C	T^T	T[p]Py
0	24.2	3.6	0.87	0.0	0.0	0.00	0	0	0
5	25.6	3.4	0.87	0.2	0.6	0.60	37	55	110
15	28.0	2.5	0.70	1.8	2.6	0.90	265	191	132
30	29.3	2.8	0.82	2.3	3.3	0.84	396	284	145
45	30.3	2.6	0.79	2.6	3.8	0.80	431	315	133
60	32.7	2.7	0.88	2.4	3.9	0.90	444	360	166
90	31.6	2.9	0.92	2.5	4.1	0.73	483	396	141
120	29.3	2.9	0.85	2.9	4.8	0.77	518	428	137
sham-irradiated	24.4	3.1	0.76	0.0	0.0	0.00	0	0	0

TABLE XIV Accumulation of UV lesions in excision fragments isolated from post-UV (40 J/m^2) incubated and sham-irradiated *E. coli* (AB1157). T^T and T^C refer to all thymine-thymine and thymine-cytosine containing cyclobutane dimers, respectively. T[p]Py refers to T[p]T + T[p]C (6-4) photoproducts. Calculations are based on the assumptions that the genome size of *E. coli* is 4.6 x 10^6 bp and the thymidine content is 0.25 (Lewin, 1985); therefore, the number of thymidine residues per *E. coli* genome equals 2.1 x 10^6. All other assumptions have been presented in Table III of Chapter 1 (page 43).

repair time (min)	% of radioactivity in analyzed sample representing various cyclobutane dimers				% of T^C cyclobutane dimers		% of T^T cyclobutane dimers		% of total cyclobutane dimers	
	d-T<p>C	dT<>dC	d-T<p>T	dT<>dT	intact	modified	intact	modified	intact	modified
0	0.00	0.00	0.00	0.00	0	0	0	0	0	0
5	0.12	0.08	0.29	0.30	60	40	49	51	52	48
15	1.27	0.54	2.00	0.63	70	30	76	24	74	26
30	1.63	0.64	2.44	0.85	72	28	74	26	73	27
45	1.80	0.80	2.57	1.26	69	31	67	33	67	32
60	1.70	0.71	2.44	1.42	71	29	63	37	66	34
90	1.72	0.76	1.87	2.25	69	31	45	55	54	46
120	1.77	1.15	1.57	3.21	61	39	33	67	43	57
sham-irradiated	0.00	0.00	0.00	0.00	0	0	0	0	0	0

TABLE XV Distribution of intact and modified cyclobutane dimers contained in excision fragments isolated from post-UV (40 J/m^2) incubated and sham-irradiated *E. coli* (AB1157).

strain	relevant genotype	repair time (hr)	% of total dpm in TCA-soluble	% dpm retained after dialysis	% of total radiolabel analyzed	% Thy involved in UV lesions in analyzed fraction			UV lesions excised per cell		
						T^C	T^T	T[p]Py	T^C	T^T	T[p]Py
AB1157	wild-type	0	24.2	3.6	0.87	0.00	0.00	0.00	0	0	0
		2	29.3	2.9	0.85	2.92	4.78	0.77	521	427	137
GW3198	AB1157 umuC	0	15.4	21.5	3.31	0.01	0.09	0.04	7	63	28
		2	13.6	27.0	3.67	0.65	1.49	0.22	501	574	170
GW2727	AB1157 lexA	0	21.8	6.4	1.83	0.00	0.00	0.00	0	0	0
		2	24.6	5.8	1.92	1.25	2.17	0.34	504	437	137
DL184	AB1157 recBC	0	18.6	12.6	2.34	0.00	0.01	0.11	0	5	54
		2	13.5	14.2	1.92	1.05	2.21	0.51	423	446	206
JC9937	wild-type	0	35.7	15.7	5.60	0.00	0.01	0.00	0	12	0
		2	34.6	15.6	5.40	0.44	0.78	0.12	499	442	136
SMR374	JC9938 recA	0	33.4	16.4	4.44	0.00	0.01	0.03	0	9	28
		2	44.9	16.2	6.18	0.24	0.47	0.13	311	305	169

TABLE XVI Accumulation of UV lesions in excision fragments from post-UV (40 J/m^2) incubated *E. coli* strains characterized by different genetic backgrounds. Note: The values at 0 hr post UV are assumed to be background.

strain	relevant genotype	% of radioactivity in analyzed fraction representing the various cyclobutane dimers				% of T^C cyclobutane dimers		% of T^T cyclobutane dimers		% of total cyclobutane dimers	
		d-T<p>C	dT<>dC	d-T<p>T	dT<>dT	intact	modified	intact	modified	intact	modified
AB1157	wild-type	1.77	1.15	1.57	3.21	61	39	33	67	43	57
GW3198	umuC	0.61	0.04	0.57	0.92	94	6	38	62	55	45
GW2727	lexA	0.95	0.30	0.83	1.34	76	24	38	62	52	48
DL184	recBC	0.84	0.21	1.18	1.03	80	20	53	47	62	38
JC9937	wild-type	0.32	0.12	0.33	0.45	73	27	42	58	53	47
SMR374	recA	0.20	0.04	0.33	0.14	83	17	70	30	75	25

TABLE XVII Distribution of intact and modified cyclobutane dimers contained in excision fragments isolated from various post-UV (40 J/m^2) incubated (2 hr) *E. coli* strains.

strain	relevant genotype	repair time (min)	% dpm retained after dialysis	% total radioactivity representing each photoproduct				
				d-T<p>C	d-T<p>T	T[p]Py	dT<>dC	dT<>dT
TK603	uvrA	5	7.5	0.32	0.73	0.11	0.04	0.13
		120	10.3	0.22	0.54	0.08	0.04	0.10
		sham-irradiated	6.7	0.00	0.00	0.01	0.00	0.01
TK610	uvrA; umuC	5	8.2	0.30	0.68	0.11	0.04	0.12
		120	9.4	0.26	0.59	0.09	0.04	0.11
		sham-irradiated	7.5	0.00	0.00	0.01	0.00	0.01

TABLE XVIII Percentage of radioactivity representing the various classes of lesions in genomic DNA isolated from post-UV (40 J/m^2) incubated and sham-irradiated excision-deficient strains of *E. coli*.

strain	relevant genotype	repair time (min)	total number of each lesion in genomic DNA				
			d-T<p>C	d-T<p>T	T[p]Py	dT<>dC	dT<>dT
TK603	uvrA	5	504	575	173	63	102
		120	476	584	173	87	108
		sham-irradiated	0	0	14	0	7
TK610	uvrA: umuC	5	517	585	189	69	103
		120	513	582	178	79	109
		sham-irradiated	0	0	16	0	8

TABLE XIX UV photoproducts remaining in genomic DNA isolated from two repair-deficient post-UV (40 J/m^2) incubated *E. coli* strains.

strain	relevant genotype	repair time (min)	T^C dimers		T^T dimers		Py^Py dimers*	
			total number	% modified	total number	% modified	total number	% modified
TK603	uvrA	5	567	11.1	677	15.0	1244	13.3
		120	563	15.5	683	15.8	1246	15.7
		sham-irradiated	0	0	7	100.0	7	100.0
TK610	uvrA; umuC	5	586	11.8	688	15.0	1274	13.5
		120	592	13.3	691	15.8	1282	14.7
		sham-irradiated	0	0	8	100.0	8	0

TABLE XX Distribution of intact and modified cyclobutane dimers contained in the genomic DNA isolated from two repair-deficient post-UV (40 J/m^2) incubated *E. coli* strains. *This number includes the sum of T^T and T^C dimers only.

repair time							
5 min				120 min			
wild-type (AB1157)		*uvrA* (TK603)		wild-type (AB1157)		*uvrA* (TK603)	
number CBD excised	number CBD modified	number CBD in genome	number CBD modified	number CBD excised	number CBD modified	number CBD in genome	number CBD modified
92	43	1244	165	946	488	1246	195

TABLE XXI A comparison of the number of cyclobutane dimers which are modified and contained within the genomic DNA of the excision-deficient strain, *uvrA*, to the number found in excision fragments of a wild-type strain. N.B. Numbers displayed are a total of T^C + T^T dimers as presented in Tables XIV, XV, and XVIX.

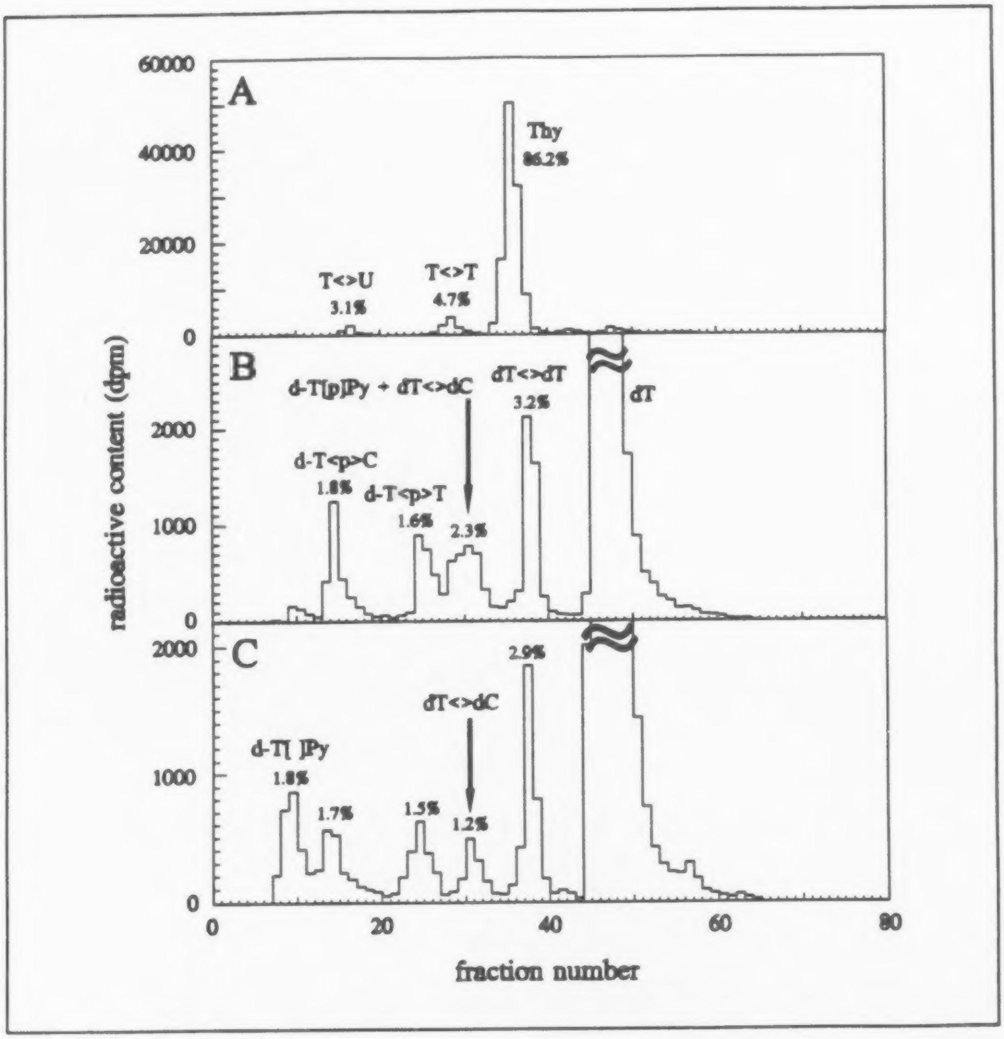


FIGURE 28 Reverse-phase HPLC radioactivity profiles of excision fragments isolated from post-UV (40 J/m^2) incubated (2 hr) wild-type *E. coli* (AB1157) cells. **A.** Formic acid hydrolysis. **B.** Enzymatic hydrolysis. **C.** Alkaline hydrolysis. Numbers indicted on chromatograms refer to % of total radioactivity in each major peak.

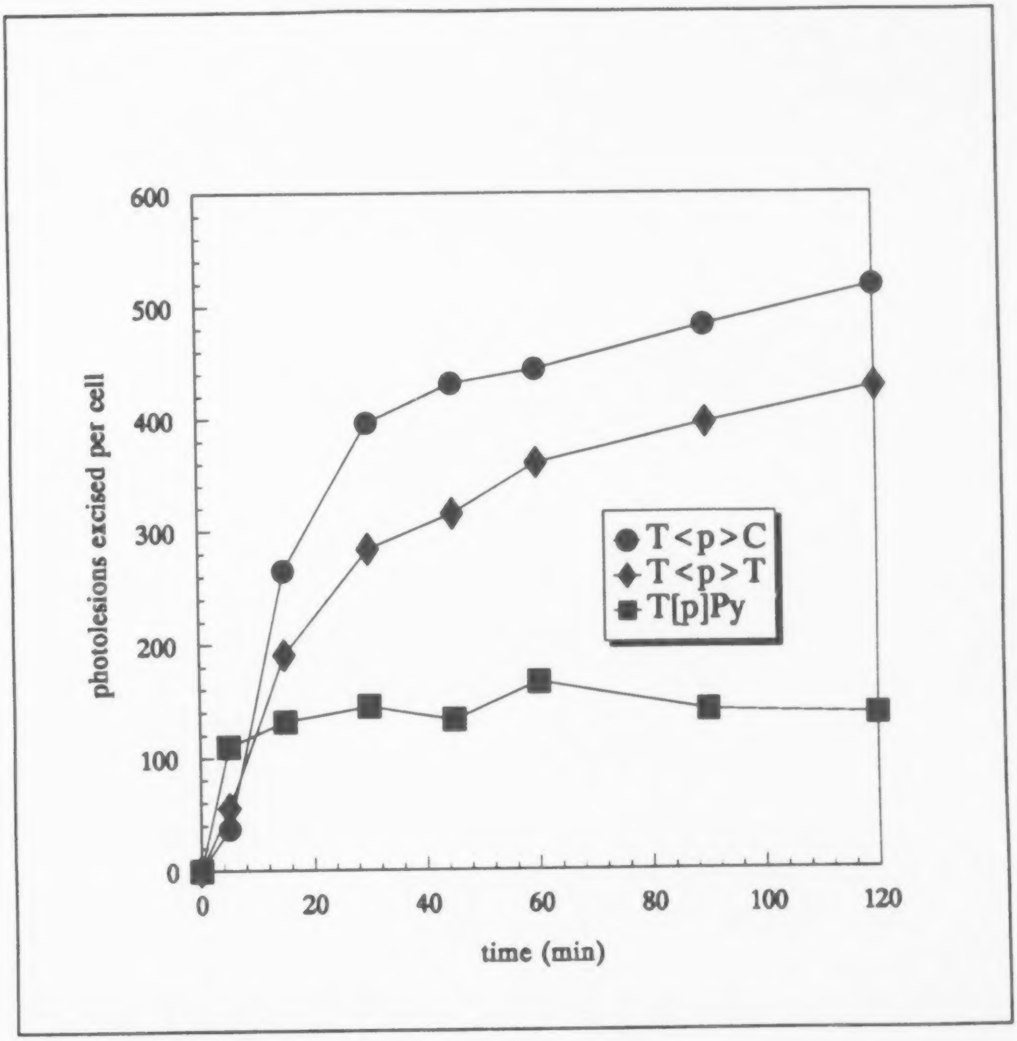


FIGURE 29 Repair of cyclobutyl dimers and (6-4) photoproducts, following 40 J/m^2 of 254-nm light, in wild-type *E. coli* (AB1157).

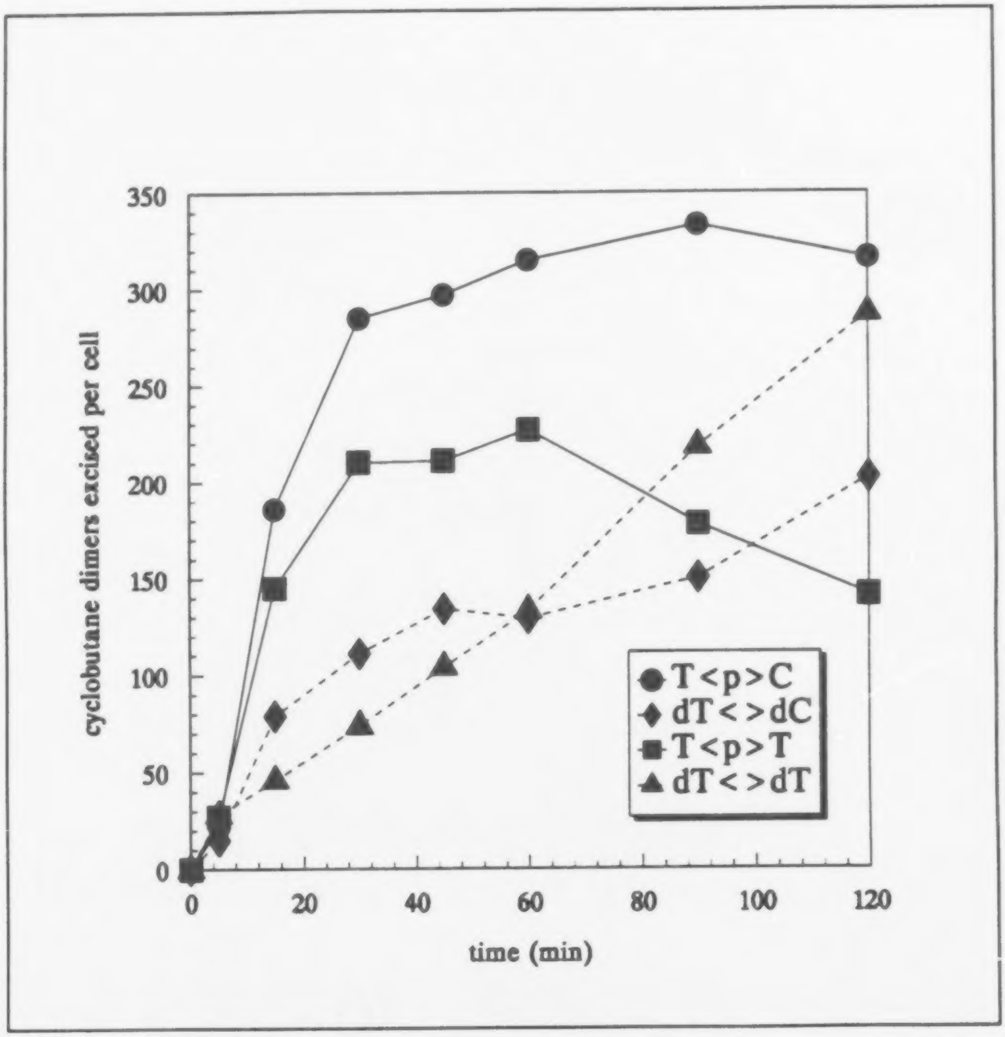


FIGURE 30 Accumulation of intact and modified cyclobutane dimers in excision fragments from post-UV (40 J/m^2) incubated wild-type *E. coli* (AB1157). Solid lines show intact dimers. Broken lines depict modified dimers.

CHAPTER EIGHT - THE DEVELOPMENT OF AN *IN VITRO / IN VIVO* SYSTEM TO ASCERTAIN THE CELLULAR FUNCTION OF INTRADIMER PHOSPHODIESTERASE ACTIVITY.

INTRODUCTION

As has been outlined in the previous chapters of this dissertation, both prokaryotic and eukaryotic cells appear to harbor intradimer phosphodiesterase activity. The cellular function of this enzyme remains obscure. To address this question, an *in vitro / in vivo* system to monitor the activity of several cellular processes on a modified dimer-containing substrate has been developed. This system is explained in detail below, but, in short, employs a pGEM vector containing (i) no lesion, (ii) a cyclobutane dimers at a defined position within the *lacZ* gene, or (iii) a cyclobutane dimer, with its interpyrimidine phosphodiester linkage severed, at the same location. This vector system also contains relevant control sequences which readily allow assessment of *in vitro* replication and transcription across the intact or modified lesion, with either prokaryotic or mammalian polymerases, as well as the activity of the UvrABC exonuclease, DenV, and other available repair enzymes. It can also be easily adopted to *in vivo* studies in *E. coli*.

This scheme was designed with the initial intent of testing the ability of DNA and RNA polymerases to bypass a cyclobutane dimer with a cleaved intradimer phosphodiester bond. Cyclobutane dimers, in their intact state, are well documented polymerase-blocking lesions in both prokaryotes and eukaryotes (Caillet-Fauquet *et al.*, 1977; Villani *et al.* 1978; Moore *et al.*, 1981; Vos and Rommelaere, 1982; Griffiths and Ling, 1989; Selby and Sancar, 1990). The premise that dimer modification may alleviate this barrier is based on a combination of observations. Many of these have been discussed in previous chapters, but are reiterated here for congruence.

The first evidence that the intradimer phosphodiester bond of a cyclobutane dimer could be severed came from studying the unrepaired lesions which remain in the genome of post-UV incubated XP group D cells (Paterson *et al.*, 1984). Because cultured XP-D cells displayed inconsistencies in repair parameters when assayed by two different protocols (unscheduled DNA synthesis and UV endonuclease-sensitive site removal), the state of these unrepaired lesions was examined in an attempt to elucidate this discrepancy. The unexpected result, that the molecular weight of DNA extracted from post-UV incubated XP-D cells was reduced upon treatment with large amounts of photolyase, led to the hypothesis that intracyclobutane pyrimidine dimer phosphodiester bonds were ruptured. Subsequent investigation of cyclobutane dimers contained within excision fragments isolated from post-UV incubated normal cells revealed that this modification was not peculiar to the repair-deficient XP-D cells (Weinfeld *et al.*, 1986). Photochemical reversal of the cyclobutane lesions contained within these oligonucleotides liberated free thymidine and thymidine monophosphate, suggesting that these adducts were (i) often terminally located within the excision fragments and (ii) often had a severed intradimer phosphodiester bond. The proposal, at that time, invoked this cleavage as a pre-incision recognition-essential process of human DNA repair (Paterson *et al.*, 1987). However, the occurrence of both intact and modified dimers in excision fragments isolated from all types of post-UV incubated cells assayed (*E. coli*, CHO, and human; see previous chapters) argues against modification being a pre-incision event essential for recognition by the DNA incision complex.

At about this same time, it became evident that mammalian cells were much more efficient at repairing (6-4) photoproducts than cyclobutane dimers (Mitchell *et al.*, 1985). This result was used to explain the observation that CHO cells, which are virtually lacking in cyclobutane dimers removal, are no more sensitive to UV exposure than are human cells (Trosko *et al.*, 1965; Klímek, 1966; Vijg *et al.*, 1984). However, this raises the question as to how rodent cells cope with unrepaired cyclobutane dimers, as they are obviously able to continue such essential cellular processes as replication and transcription, in spite of the persistence of these polymerase-blocking lesions. A function which allows cells to overcome barriers to replication has been predicted (Vos and Rommelaere, 1982). In addition, given the ability of cyclobutane dimers [but not (6-4) photoproducts (see Chapter 2)] to be chemically altered by interpyrimidine phosphodiester cleavage, the conceivable hypothesis, that severage of the intradimer phosphodiester linkage alleviates the distortion caused by a cyclobutyl bridge and thereby effects accurate base pairing, was formulated. Reformation of precise hydrogen bonding would remove the block to polymerases and allow a cell to continue vital processes even though the lesions remain in the genome. The predicted presence of modified dimers in the genome of post-UV incubated CHO cells has been documented (Pirsel *et al.*, 1989).

At about this same time, the preferential repair of cyclobutane dimers present in transcriptionally active sequences was described (Bohr *et al.*, 1985; Mellon *et al.*, 1986; Bohr *et al.*, 1987; Mellon *et al.*, 1987) and hypothesized to account for the high survival, given depressed efficiency of cyclobutane dimer repair, in CHO cells. Since that time, preferential repair has been characterized in both eukaryotic and prokaryotic systems (Mellon *et al.*, 1986;

Mellon and Hanawalt, 1989). The method used to discern this specific mode of repair is important to an alternative hypothesis presented here, and will therefore be discussed briefly (see also Figure 31). We have postulated that, what had been described as preferential repair may, in reality, be cyclobutane dimer modification.

In the method developed by Bohr *et al.* (1985), cyclobutane dimer repair in different regions of the genome is ascertained using a modified Southern blot. Strand-specific probes are generated for both transcribed and non-transcribed strands of a transcriptionally active sequence, and well as for a quiescent genomic region. Cells are irradiated with a fluence sufficient to induce, on average, one cyclobutane dimer per probed region. Nonreplicated genomic DNA is then isolated from these UV-treated cells either immediately, or following various post-UV incubation times. It is subsequently digested with a restriction enzyme and divided into two aliquots, one of which is given no further treatment, and the other is hydrolysed with the T4 UV endonuclease, DenV. This repair glycosylase/endonuclease is specific for cyclobutane dimers (Gordon and Haseltine, 1980; Nakabeppu *et al.*, 1982), producing a single-stranded break at the site of the lesion. If cyclobutane dimers are present in a probed region, DenV hydrolysis yields a reduction or disappearance (depending on the proportion of genomes containing dimers in this region) of a discrete band upon probing the Southern blot, as these restriction fragments are then no longer of a distinct length. Once dimers have been repaired, DenV can no longer reduce the molecular weight of this restriction fragment and thus a distinct band is discernable. The number of dimers present in any post-UV repair sample is calculated based on the proportion of genomes without a dimer (the zero class) and assuming a Poisson distribution.

In the initial XP-D DNA studies (Paterson *et al.*, 1984) which elucidated the existence of intradimer phosphodiesterase cleavage, it was found that photolyase would only bring about photoenzymatic reversal of a modified cyclobutane dimer if large quantities (10-fold excess) of the enzyme were used. It was therefore questioned whether or not the protocol, employed to uncover preferential repair, had taken into account that the enzyme (DenV) used to discern cyclobutane dimer sites may not recognize the two molecular configurations (intact versus modified cyclobutane dimers) with the same efficiency. Members of our laboratory, therefore, repeated these experiments and extended the analysis by exploring the consequence of photoenzymatic reversal. This work detected the transient appearance of modified cyclobutane dimers in transcriptionally active genes of normal human fibroblasts at very early repair times (Middlestadt *et al.*, 1990). Furthermore, in XP-D cells, these altered lesions accumulated specifically in actively transcribed genomic regions. This, then, implies that a portion, if not all, of preferential repair is actually intradimer phosphodiester cleavage.

To further address this postulate and to provide a direct means of assessing the effect of modified dimers in replication and transcription, an *in vitro* system has been developed. Although these substrates have many *in vitro* and *in vivo* applications (to be discussed in the final chapter of this dissertation), to date only preliminary *in vitro* replication and transcription experiments have been executed, as well as a detailed study of the ability of DenV to recognize the modified adduct. These results confirm the anticipated outcome that DenV does not recognize a cyclobutane dimer with a severed intradimer phosphodiester linkage, questioning the interpretation of the accumulated data on preferential repair. Furthermore, preliminary inquiries of polymerase function on encountering this adduct suggest that, although interpyrimidine phosphodiester cleavage plays no apparent role in replication bypass, it allows at least the viral SP6 RNA polymerase to synthesize past this otherwise transcription-blocking lesion.

EXPERIMENTAL PROCEDURES

For more detailed experimental procedures, see Appendix B, page 278.

Materials. Oligonucleotides were purchased from the DNA synthesis facility in the Department of Microbiology, University of Alberta. The pGEM-7Zf(-) vector, *Nsi*I, *Sph*I, *Apa*I, *Acy*I, T4 ligase, T4 DNA polymerase, reverse sequencing primers, *E. coli* DNA polymerase I, SP6 RNA polymerase (supplied with concentrated buffer and dithiothreitol), and RNase inhibitor, RNasin, were all obtained from Promega (Madison, WI). *Pvu*II, T4 polynucleotide kinase, calf alkaline phosphatase, nucleotides and deoxynucleotides were acquired from Pharmacia (Uppsala, Sweden). All other chemicals were purchased from the BDH Chemicals (Toronto, ON), unless otherwise stated. All media and buffers were prepared as outlined in Sambrook *et al.* (1989).

Substrate construction.

Preparation of pGEM vector sequences. The structure of the M13-derived vector, pGEM-7Zf(-), is illustrated in Figure 32. DH5α competent *E. coli* cells (BRL Inc., Burlington, ON) were transformed with the pGEM plasmid. This was accomplished by incubating 100 µl of competent cells with 5 µl (0.05 ng) of plasmid, on ice, for 45 min. After heat-shocking (42°C for 45 seconds), 1 ml of LB medium was added, and the mixture incubated for 1 hr at 37°C, without shaking. Dilutions (1/10, 1/50, 1/100) were then plated on LB agar plates containing 100 µg/ml ampicillin. After overnight incubation at 37°C, an Amp^R colony was selected and used to inoculate 10 ml of LB. This was grown overnight at 37°C.

In the morning, 1 ml of the overnight culture was added to 1 *l* of LB and incubated in a 2-*l* flask, with aeration, at 37°C. After the culture had reached an OD_{600} of 0.6, cells were harvested, and subsequently resuspended in 30 ml of a solution containing 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0), and 10 mg/ml lysozyme, and incubated on ice for 10 min. Seventy ml of a second solution, containing 0.2 N NaOH and 1% SDS, was added to the cell suspension and incubation continued for an additional 10 min. The cell lysate was then supplemented with 50 ml of 3 M KAc (pH 4.8) and left on ice for 15 min. Bacterial DNA and proteins were pelleted by centrifugation at 10,000*g* for 15 min. The supernatant was filtered through gauze, and the plasmid DNA precipitated from the now clear liquid with 2 volumes of ice-cold 100% ethanol. After recovery by centrifuging at 10,000*g* for 10 min, the pellet was resuspended in 4 ml TE. The suspension was added to 8.2 ml of a saturated CsCl solution (in TE) and 400 µl of ethidium bromide solution (10 mg/ml). Gradient formation was achieved by spinning at 55,000 rpm in a fixed angle rotor at 20°C for 48 hr. The lower band (CCC DNA) was recovered, and the ethidium bromide was removed by extracting 5 times with water-saturated n-butanol. CsCl was removed by dialysis against TE. The final concentration of the purified plasmid was 7.7 mg/ml.

After confirming that either *Nsi*I or *Sph*I could completely digest the pGEM-7Zf(-) vector to a re-ligatable product in a buffer system recommended for use with *Nsi*I, these two restriction enzymes were used to double-digest the vector sequence in the same reaction mixture. Approximately 560 µg/reaction of pGEM vector was restricted with *Nsi*I and *Sph*I (Promega) in a 1-ml reaction containing 10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol, 0.1 mg/ml BSA, 200 units *Nsi*I, and 200 units *Sph*I. Digested samples were inspected by agarose gel electrophoresis.

*Nsi*I and *Sph*I double-digestion of pGEM-7Zf(-) generates 83- and 2917-bp fragments. Since the larger vector sequence was required for subsequent steps, and because it was important to insure that in later ligation reactions the small vector sequence was not present to regenerate the starting molecule, these two fragments were separated by gel filtration chromatography. A 30-ml sepharose CL-6B (Pharmacia) column was prepared and equilibrated with 0.1 M Tris-HCl (pH 7.5), 10 mM EDTA. The flow rate was set at 0.1 ml/min using a peristaltic pump and 5-min fractions were collected. The OD_{260} of each fraction was determined and aliquots comprising the first UV-absorbing peak, which contained the 2917-bp fragment [fractions 19-26 (see Table XXIX)], were pooled and dialysed against ddH_20. The final concentration of this material was 104 µg/ml.

To ensure that the 83-bp fragment has been removed, re-ligation of the gel filtration-purified 2917-bp vector sequence was tested. This ligation reaction employed the same procedure outlined below for attaching the oligonucleotides to the 2917-bp vector sequence. As a control for ligation, unfractionated *Nsi*I/*Sph*I restricted pGEM was also re-ligated.

Oligonucleotides inserted into the pGEM vector. The five oligonucleotides purchased from the DNA synthesis laboratory were designated AGA-4, AGA-6, AGA-7, AGA-8, and AGA-9. AGA-4 is a 5'-phosphorylated 12-mer of sequence 5'-p-GTCCGGAGTGCA. AGA-6 is a non-phosphorylated 11-mer with the sequence 5'-GCAAGTTGGAG. AGA-7 is a 5'-phosphorylated 23-mer used as the complementary strand to AGA-4 and AGA-6. It has the sequence 5'-p-CTCCGGACCTCCAACTTGCCATG. AGA-8 and AGA-9 together comprise AGA-6. AGA-8 is a 5'-phosphorylated 5-mer of sequence 5'-p-TGGAG. AGA-9 does not have a terminal phosphate and has the sequence 5'-GCAAGT (see Figure 33). Approximately one µmol of each oligonucleotide was synthesized, and all were received in a dried state. All were dissolved in 1 ml of ddH_2O, and the concentration determined by further diluting a 5-µl aliquot up to 1 ml with ddH_2O and measuring the OD_{260}. The concentrations of the oligomers varied between 0.7 and 3.4 mg/ml.

Before AGA-4 and AGA-7 were subjected to any further manipulations, they were purified on a NAP-5 column (Pharmacia) which is a prepacked disposable gel filtration column, containing Sephadex G-25. The column was first equilibrated with 3 volumes of TE buffer, after which 0.5 ml of an oligonucleotide solution was applied, and the first 0.5 ml which eluted was discarded. The purified oligomer was then eluted with 1 ml of TE. Small molecules, such as salts, are left on the column if only a 1.5-ml volume is allowed to pass through the column matrix. Because further manipulations of AGA-6, AGA-8, and AGA-9 involved HPLC purification steps, these oligomers were not cleaned on NAP-5 columns (see below).

Production and purification of cyclobutane dimer-containing AGA-6. The AGA-6 oligomer contains only one dipyrimidine site, adjoining TT residues. This is, therefore, the only position where a cyclobutane dimer can be formed. Dimer induction was achieved by irradiating AGA-6 with 10 kJ/m^2 of 254-nm light in the following manner. Approximately 300 nmol of AGA-6 was irradiated by adding 30 nmol at a time to a 150-mm tissue culture dish in a total volume of 20 ml. This was placed under a germicidal lamp which emits 97% of its radiant energy at the 254-nm wavelength (see Chapter 2, page 15, 258, for specifications). The solution was stirred continuously to assure uniform irradiation.

Because cyclobutane dimers formation / photochemical reversal occurs in equilibrium at very high doses of 254-nm light, only a minority of the irradiated molecules would contain a cyclobutane dimer. [For example, in poly(dT), a dose of 10 kJ/m_2 of 254-nm light dimerizes approximately 35% of the thymine. In native human DNA, the maximum yield of T^T dimers which can be achieved with 254-nm light is approximately 7% (Rahn and L.C. Landry, 1971).] The irradiated oligomers, then, were purified by HPLC chromatography, with slight modifications to the procedure of Banerjee *et al.* (1988). Separation of the T^T-containing molecule from the parent molecule and other photoproduct-containing molecules was achieved on a Nova-Pak C_{18} reverse-phase column (250 x 4.6 mm i.d.; Waters, Mississauga, ON). The column was equilibrated with 7.5% acetonitrile in 0.1 M triethylammonium acetate. The separation protocol consisted of a linear gradient from 7.5% to 9% acetonitrile over the first 20 min, followed by a linear gradient to 75% acetonitrile over the succeeding 10 min, and subsequently a return to initial conditions in 10 min. A flow rate of 1 ml/min was maintained throughout, and 80 x 0.5-min fractions were collected. The fractions containing the parent molecule were pooled, as were those suspected of containing the oligomer with a T^T dimer, and both pools were taken to dryness in a rotoevaporator (Büchi, Switzerland). Each pooled sample was resuspended in ddH_2O and dialysed against water to reduce the high concentration of triethylammonium acetate. Purification was repeated three times in total to ensure that all other types of molecules had been removed.

The identity of the peak representing the T^T-containing 11-mer was confirmed by both photochemical and photoenzymatic reversal. For the former confirmation procedure, 1 µg of the purified T^T-containing 11-mer was diluted to a final volume of 1 ml with ddH_2O. This was placed in a 40-mm tissue culture dish and exposed to a fluence of 5.5 kJ/m^2 of 254-nm light. The irradiated material was then reinjected onto the HPLC and analyzed by the same gradient protocol as described above. For photoenzymatic reversal, 1 µg of the purified oligomer was diluted into a 200-µl reaction mixture containing 50 mM Tris (pH 7.5), 125 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol and 10 µg of photolyase (purified in our laboratory using the cloned gene supplied by Dr. Aziz Sancar). This mixture was placed under fluorescent light for 1 hr at room temperature and subsequently analyzed by HPLC reverse-phase as indicated above.

Following HPLC purification of AGA-6 and its T^T-containing derivative, both were 5' phosphorylated. This was executed in two ways: by kinasing with (i) unlabelled ATP to generate a population of molecules for eventual insertion into the pGEM vector sequence, or (ii) [γ-^{32}P] ATP. This latter post-labelling procedure simplified further examination of these molecules. Very small quantities could now be analyzed as there was no longer dependence on UV-absorption profiles which require significant amounts of material and often present extraneous peaks unrelated to the compound(s) of interest.

Nonradioactive phosphorylation of the dimer-containing oligonucleotide or parent molecule was accomplished in the following manner. Two nmol of oligomer were kinased in a 1-ml reaction mixture consisting of 10 mM Tris acetate (pH 7.0), 10 mM magnesium acetate, 50 mM potassium acetate, 50 nmol unlabelled ATP, and 0.2 units/µl of T4 polynucleotide kinase. The reaction was incubated for 1 hr at 37°C prior to further purification by HPLC (see below). This procedure was repeated 10 times in total for each AGA-6 and T^T-containing AGA-6. For radioactive end-labelling, 50 pmol of oligomer were kinased in a 50-µl volume containing the same components as indicated above, except instead of unlabelled ATP, 5 µCi of [γ-^{32}P] ATP (1 mCi/ml; 3,000 Ci/mmol; Amersham, Burlington Heights, IL) were present.

The 5'-phosphorylated population of T^T-containing AGA-6 was purified from the unphosphorylated molecules using HPLC chromatography. The same Nova-Pak reverse-phase C_{18} column was employed as described above, but the gradient scheme varied slightly. The initial conditions (6% acetonitrile in 0.1 M triethylammonium acetate) were maintained for 2 min. A linear gradient from 6% to 9% acetonitrile over 18 min was then followed by a linear gradient to 70% acetonitrile over the next 10 min, and finally, a return to initial conditions over 10 min. The fractions containing the phosphorylated molecule were pooled, taken to dryness in a rotoevaporator, resuspended in ddH_2O, and dialysed overnight against water. The final concentration of 5'p-AGA-6 and T^T-containing 5'p-AGA-6 were 10 pmol/µl and 2 pmol/µl, respectively.

Isolation of oligonucleotides with a modified cyclobutane dimer. Initial attempts to generate an oligomer with a modified dimer entailed treatment of the HPLC-purified oligomers with the human intradimer phosphodiesterase (IDP) activity, isolated from human liver by Dr. Michel Liuzzi and John Chan in our laboratory. Radioactively end-labelled AGA-6 and T^T-containing AGA-6 were utilized in these experiments. One pmol (~8 x 10^5 cpm) of these substrates were each (individually) incubated in a total of 50 µl at 37°C for 1 hr in the presence of 25 mM NaAc (pH 5.5), 2.5 mM $MgCl_2$, 1 mM β-mercaptoethanol, and 0.5 µg IDP. After heating to 100°C for 10 min, an aliquot (~8 x 10^4 cpm) of the reaction mixture was analyzed by HPLC, using the same gradient system as employed for the isolation of phosphorylated substrate derivatives. As this procedure revealed no evidence of alteration in the structure of the IDP-treated substrate, the reaction mixture was analyzed by polyacrylamide gel electrophoresis (see Chapter 2, page 19 for details) following (i) no further treatment, (ii) photoenzymatic reversal, and (iii) photochemical reversal. In parallel, T^T-containing AGA-6 which had not been exposed to IDP, IDP treated and untreated AGA-6, and dimer-containing substrate which and been IDP treated in the presence of photolyase in the dark, were analyzed after the same procedures. Photolyase treatment (2 µg enzyme/pmol DNA) and photochemical reversal were carried out in the same manner as described above.

Generation of a modified dimer in AGA-6 was unsuccessful using IDP. Further experimentation indicated that this human enzyme was not active on double-stranded and single-stranded oligonucleotides of less than 25 base pairs or nucleotides (data not shown). Therefore, a presumably similar structure was constructed by photoligating two smaller oligonucleotides (Lewis and Hanawalt, 1982). Prior to this experiment, a small amount of AGA-8 and of AGA-9 were each end-labelled to allow easier assessment of the reaction outcome. Since AGA-8 had been purchased in a 5'-phosphorylated form, this was first hydrolysed with calf alkaline phosphatase. Fifty pmol of AGA-8 was dephosphorylated in a 50-µl reaction containing 50 mM Tris-HCl (pH 8.5), 0.1 mM EDTA, and 1 unit phosphatase, for 1 hr at 37°C. After heat inactivation (75°C for 10 min), and phenol/chloroform extraction, Tris-acetate, magnesium acetate, and potassium acetate were added to a final concentration of 10 mM, 10 mM, and 50 mM, respectively. Upon the addition of 2 µCi [γ-^{32}P] ATP and 20 units of T4 polynucleotide kinase, the reaction was incubated for 1 hr at 37°C. AGA-9 was end-labelled in the same manner, but without initial phosphatase treatment. The retention time on the reverse-phase HPLC system being used was established for both molecules.

To allow photoligation of the 3'-thymidine of AGA-9 to the 5'-thymidine of

AGA-8, these oligonucleotides were juxtapositioned by their hybridization to AGA-7. In the initial reactions, 2 pmol of each oligonucleotide (with AGA-9 and/or AGA-8 containing a [^{32}P]-labelled terminal phosphate) was hybridized in a 100-µl reaction volume containing 50 mM Na Phosphate (pH 6.8) and various NaCl concentrations ranging from 50 mM to 2 M. This would establish not only a marker for the AGA-9<>AGA-8 photoligation product (and its 5' phosphorylated derivative), but also the most appropriate NaCl concentration to facilitate annealing and, therefore, generate the maximum yield of this species. The reaction mixture was heated to 65°C for 10 min, and subsequently allowed to cool to room temperature. The hybridization reaction was then left at 4°C overnight. In the morning, the hybridized oligonucleotides were irradiated, one ice, with 254-nm light at a fluence of 5.5 kJ/m^2. The resulting products were analyzed by reverse-phase HPLC adopting the same procedure as had been used for separating AGA-6 from its dimer-containing derivative. The identity of novel peaks (photoligated AGA-9<>AGA-8 with or without a terminal phosphate on AGA-9) was confirmed by regeneration of the parent oligonucleotides upon photochemical reversal (5.5 kJ/m^2) of an aliquot of this material isolated from the HPLC.

Having established reaction conditions, 360 nmol of AGA-9 and 90 nmol each of AGA-8 and AGA-7, supplemented with 2 pmol of end-labelled AGA-8, were mixed in a 1-ml reaction volume containing 50 mM Na phosphate (pH 6.8) and 1 M NaCl (the empirically determined optimal concentration of NaCl). The molecules were allowed to hybridize as outlined above, and were subsequently irradiated with 5.5 kJ/m^2, and the species of interest isolated by HPLC. As HPLC was performed at room-temperature, oligonucleotides would be single-stranded during this purification step. This procedure generated 25 µg of photoligated AGA-9<>AGA-8.

Following purification of this artificially constructed, modified cyclobutane dimer-containing oligonucleotide, it was subjected to terminal phosphorylation, using the same procedure as employed for T^T-containing AGA-6, and subsequently purified by HPLC. Fractions of interest were pooled, taken to dryness in a rotoevaporator, resuspended in ddH$_2$O, and dialysed overnight against water. The final concentration of the modified dimer-containing 11-mer was 3 pmol/µl.

Ligation of oligonucleotides into the pGEM vector and subsequent purification. The low yield of CCC DNA upon ligation of the various oligonucleotides with the 2917-bp pGEM fragment quickly made it evident that pre-hybridization of the molecules was necessary. For this purpose, in a reaction buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 10 mM dithiothreitol, 16 pmol of the 2917-bp pGEM-7Zf(-) fragment were mixed with 160 pmol each of AGA-4 and AGA-7 along with 160 pmol of one of (i) AGA-6, (ii) T^T-containing AGA-6, or (iii) AGA-9<>AGA-8 (final volume, 20 ml). The mixture was heated to 65°C in a circulating water bath for 10 min. The waterbath was then adjusted to 14°C and the hybridizing mixture was allowed to cool gradually. The target temperature of 14°C was maintained for 1 hr before adding ATP to a final concentration of 16 mM, and 15 units of T4 ligase. Incubation was then continued overnight at 14°C.

In the morning, the ligation mixture was phenol/chloroform extracted, and the DNA ethanol precipitated. This was left overnight at -20°C. The DNA pellet was collected by centrifugation for 10 min (4°C) at 5000 rpm, the ethanol was decanted, and the DNA dried under vacuum in a SpeedVac Concentrator (model SVC 100H; Savant Instruments, Inc., Farmingdale, NY). The DNA was then resuspended in 100 µl of ddH_2O, supplemented with 50 µl loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol), loaded (20 µl per lane) onto a 0.8% low melt agarose (ultraPURE; BRL) gel containing 0.5 µg/ml ethidium bromide, and electrophoresed overnight (4°C, 20 V), using TAE buffer. In the morning, bands were visualized by UV light, and the covalently closed circular DNA band was cut from the gel. The DNA was then purified by electroelution. This was accomplished by placing the gel slice in a 2.5-cm wide dialysis bag (3 kDa MW cut-off), with approximately 500 µl of TAE. After placement in a electrophoresis tank, a current of 150 V was applied for 3 hr. The polarity was then reversed for 1 min, freeing the electroeluted DNA from the wall of the dialysis membrane. The buffer surrounding the gel slice was transferred to a 1.5-ml polyurethane tube and subjected to phenol/chloroform (Sambrook *et al.*, 1989) to remove both ethidium bromide and gel contaminants. The DNA was subsequently ethanol precipitated in the presence of 1 M ammonium acetate. After collection by centrifugation, the pellet was dried and resuspended in 500 µl ddH_2O. The DNA substrates were then concentrated and further purified using a Centricon-10 concentrator (Micon, Beverly, MA). Each sample was applied to the top reservoir of the device and spun at 4800*g* for 15 min in a fixed angle rotor (Sorvall SA-600). The sample was washed twice with 500 µl ddH$_2$O before inverting the unit and collecting the concentrated, cleaned sample by spinning at 600*g* for 2 min. Purified substrates were stored at 4°C until further use. Approximately 10% (or 1 ng) of the input DNA was recovered as CCC substrate for each reaction.

For simplicity, the three substrates will be referred to by code names: m-pGEM contains the unaltered oligonucleotides, AGA-4, AGA-6, and AGA-7; ID-pGEM harbors a cyclobutane dimer at the unique TT sequence in AGA-6; MD-pGEM contains a modified dimer, constructed by photoligating AGA-9 to AGA-8, at the same site.

Assessment of the activity of DenV on unaltered and modified cyclobutane dimers.

DenV hydrolysis of UV-irradiated CCC plasmid DNA. The specific activity of three independent preparations of DenV (prepared in our laboratory) was ascertained prior to further experimentation. For this purpose, 70 µg of pGEM-7Zf(-) was diluted to 1 ml with ddH$_2$O and placed in a 40-mm tissue culture dish. The solution was then exposed, with continuous mixing, to 2 kJ/m^2 of 254-nm light. At an induction rate of 1.2 x 10^{-5} cyclobutane dimers/nt/J/m^2, this would generate, on average, 72 cyclobutane dimers per plasmid molecule. DenV was then tested on this, as well as on non-irradiated plasmid diluted to the same concentration.

In a total reaction volume of 20 µl containing 32 mM Tris-HCl (pH 7.5), and 9.6 mM EDTA, 350 ng of irradiated or non-irradiated plasmid DNA was incubated in the presence of varying amounts of 3 different DenV preparations for 30 min at 37°C. NaOH was subsequently added to a final concentration of 0.2 N and the mixture held for a further 30 min at 37°C. The reaction was then supplemented with SDS to a final concentration of 0.01%. Prior to analysis by agarose gel electrophoresis, the mixture was supplemented with 2.5 µl loading dye. Electrophoresis was carried out using a 0.8% agarose gel, containing 0.5 µg/ml ethidium bromide, at 50 V for 2 hr at room temperature. Specific incision by DenV could be detected as a conversion from CCC to open circle DNA for the UV-irradiated substrate, without effect on the untreated plasmid. The active DenV preparation was ascertained to contain ~70 units/µl (see Appendix C).

DenV hydrolysis of pGEM-7Zf(-) containing at a defined site (i) no lesion, (ii) an

intact cyclobutane dimer, or (iii) a cyclobutane dimer with a severed intradimer phosphodiester backbone. Once an active preparation of DenV had been confirmed, the aforementioned protocol was performed on the three constructed CCC substrates. Because of the limited quantities of these substrates, ~500 fmol of each was restricted with PvuII (Pharmacia), generating 373- and 2567-bp fragments, treated with calf alkaline phosphatase to remove terminal phosphate groups, and end-labelled using T4 polynucleotide kinase and [γ-^{32}P] ATP, as described previously. The substrates were then phenol/chloroform extracted and purified by ethanol precipitation prior to further manipulation.

In a similar manner as for UV-irradiated plasmid, approximately 15,000 cpm (~30 fmol) of m-pGEM, ID-pGEM, or MD-pGEM was incubated in a 5-µl reaction containing 32 mM Tris-HCl (pH 7.5), 9.6 mM EDTA and 35 units of DenV. After 30 min at 37°C, NaOH was added, incubation continued, and the reactions supplemented with SDS as indicated above. DenV reactions were also repeated using 700 units of DenV/30 fmol of substrate, in a total reaction volume of 20 µl. Upon the addition of 2 µl of Stop buffer (Pharmacia; consists of 0.2% bromophenol blue, 0.2% xylene cyanol, and 90% formamide), the reaction mixtures were heated to 80°C for 2 min to ensure denaturation, and 5 µl of each loaded onto a 7% denaturing sequencing polyacrylamide gel (made using 8 M urea and 1 x TAE buffer) alongside [^{32}P] end-labelled nucleotide markers. This marker DNA ladder is a HaeIII-digest of pBR322 and consists of 22 fragments ranging from 8 to 587 bp (8 / 11 / 18 / 21 / 51 / 57 / 64 / 80 / 89 / 104 / 123 / 124 / 184 / 192 / 213 / 234 / 267 / 434 / 458 / 504 / 540 / 587 bp), which become single-stranded under the electrophoresis conditions employed. Separation was achieved by applying 70 W for 2 hr. The gel was then transferred onto Whatman filter paper, wrapped with plastic film, and exposed overnight on diagnostic X-ray film (35 x 43 cm; Kodak Canada Inc., Toronto, ON). Following film development, the relative amount of DNA representing individual bands was assessed using an LKB UL Ultrascan XL Laser Densitometer (LKB-Produkter, Bromma, Sweden).

The above experiment was also repeated using m-pGEM, ID-pGEM, and, instead of MD-pGEM, IDP-treated ID-pGEM. Prior to DenV hydrolysis, approximately 15,000 cpm (30 fmol) of ID-pGEM was treated with 5 ng of human liver IDP under the conditions described above, except scaling down the quantity of IDP to 5 ng. The substrate was then purified by phenol/chloroform extraction, ethanol precipitated, and subsequently utilized in the DenV experiments.

Assessment of the *in vitro* activity of *E. coli* DNA polymerase I on unmodified and modified cyclobutane dimers.

Substrate preparation. As replication, in this system, is to begin from the reverse sequencing primer (see Figure 34), the substrate DNA was first cut at an appropriate restriction site such that *in vitro* replication would yield a product of a discrete size. Three different restriction enzymes were used in different experiments, ApaI (Promega), AcyI (Promega), and PvuII (Pharmacia). Approximately 1.2 pmol of m-pGEM, ID-pGEM, and MD-pGEM were each restricted in a 50-µl reaction containing the supplied reaction buffer, 0.1 mg/ml BSA, and 20 units of the appropriate restriction enzyme. This digested DNA was subsequently phenol/chloroform extracted and purified by ethanol precipitation.

Annealing of the reverse sequencing primer to the substrate. 50 fmol of restricted m-pGEM, ID-pGEM, or MD-pGEM was incubated in 0.4 M NaOH, in a total volume of 10 µl, for 10 min. The reaction was then supplemented with 3 µl of 3 M sodium acetate (pH 4.8), and the volume increased to 20 µl with ddH_2O. After the addition of 60 µl 100% ethanol, the mixture was incubated on dry ice for 30 min, the pellet collected by centrifugation, washed once with 70% ethanol, and taken to dryness under vacuum. The pellet was redissolved in 14 µl of annealing buffer [containing 0.1 M NaCl, 10 mM Tris-HCl (pH 7.8), and 1 mM EDTA] and 2 ng of 17-mer reverse sequencing primer (Promega). This was incubated at 37°C for 20 min and, subsequently, at room temperature for 1 hr.

***In vitro* replication.** The 14-µl mixture containing the appropriate substrate with an annealed reverse sequencing primer, was supplemented with 2.5 µl of 10 x polymerase buffer [0.6 M Tris-HCl (pH 7.5), 0.1 M $MgCl_2$, and 0.1 M β-mercaptoethanol], 2.5 µl deoxynucleotide mixture (2 mM dATP, 2 mM dGTP, 2 mM dCTP, and 100 µM dTTP), 5 µl [α-^{32}P]TTP (10 mCi/ml; 3000 Ci/mmol; Amersham), and 10 units of *E. coli* DNA polymerase I, and subsequently incubated at 12°C for 2 hr. The reaction was terminated by the addition of 5 µl of Stop buffer, and the radioactive products subsequently analyzed alongside marker DNA on a 7% denaturing sequencing polyacrylamide gel, as indicated above for the DenV reactions.

Assessment of the *in vitro* activity of SP6 RNA polymerase on unaltered and modified cyclobutane dimers.

Substrate preparation. All three substrates were prepared in the same manner as for *in vitro* replication with the following exception. Since ApaI leaves 3'-protruding single-stranded ends, these were blunt ended using T4 DNA polymerase. Prior to phenol/chloroform extraction of the restriction enzyme digest, 1 µl of deoxynucleotide solution (2 mM dATP, 2 mM dGTP, 2 mM dCTP, 2 mM dTTP) and 10 units of T4 DNA polymerase were added to the ApaI digestion mixture, and subsequently the combined mixture was incubated at 37°C for 10 min.

***In vitro* transcription using SP6 RNA polymerase.** 50 fmol of substrate was incubated in a 20-µl reaction mixture containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 40 units RNasin, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 12 µM UTP, 3 µl [α-^{32}P] UTP (10 mCi/ml; 3000 Ci/mmol; Amersham), and 15 units SP6 RNA polymerase for 1 hr at 37°C. The reaction was terminated with 5 µl stop buffer and subsequently heated for 2 min at 80 °C prior to analysis by denaturing polyacrylamide gel electrophoresis (as described above for *in vitro* replication.)

RESULTS

Choice of substrate and rationale of plasmid design. The Promega M13-derived pGEM-7Zf(-) vector (see Figure 32) was selected for the construction of intact and modified dimer-containing substrates for several reasons. The existence of a multiple cloning sequence allows insertion of oligonucleotides in a convenient manner. In addition, as this sequence is flanked by the SP6 and T7 promoters, strand-specific transcripts can easily be generated for any inserted sequence. Furthermore, as this region is within the lacZ gene, the promoter of this gene is also available for initiation of transcription. Replication can be assessed in several different ways. *E. coli ori* as well as the *f1 ori* are nearby authentic origins of replication. In addition, the availability of both the reverse sequencing primer (which binds downstream of the inserted sequence) and the forward sequencing primer (which binds upstream) allows strand-specific replication analysis. Moreover, these sequencing oligonucleotides can be used for primer extension by any eukaryotic or prokaryotic DNA polymerase as they negate the need for specific initiation sequences.

In the design of a plasmid containing a cyclobutane dimer at a specific dithymidine site, several technicalities needed to be considered. Irradiation of plasmid DNA will generate photolesions at non-specific sites. However, an oligonucleotide carrying a specific photoproduct at a unique site can be inserted into a vector, as long as the end-product can be selected. To accomplish this, it was ensured that the only CCC molecule formed in the final ligation step was the species of interest. This required cutting the plasmid with two different restriction enzymes, each with a unique site and within the multiple cloning sequence. This ensured that all vector sequences of interest were retained on the same restriction fragment. Subsequent separation of two fragments generated by this digestion provided a vector sequence which carried all of the required control elements, but which could not re-ligate to form a CCC DNA. In addition, the photolesion-containing sequence to be ligated to this vector DNA was double-stranded and possessed the same two non-identical complementary ends as did the vector fragment. This guaranteed unidirectional insertion of a single copy.

Irradiation of an oligonucleotide containing only one dipyrimidine site (and lacking TA dinucleotide sequences) will generate photolesions at this unique dipyrimidine position. However, as cyclobutane dimer formation reaches equilibrium at 5 kJ/m^2 with 254-nm light, and because (6-4) photoproducts are also induced, cyclobutane dimer-containing molecules required separation from nondimer-containing material. This was accomplished by reverse-phase HPLC, but as this methodology has a molecular weight restriction, the oligonucleotide had to be single-stranded and could not exceed 11 nucleotides. In addition, as cyclobutane dimers interfere with base-pairing, the dipyrimidine site was positioned at the middle of the oligonucleotide such that the interference of hybridization (with both opposite strand and complementary vector ends) by dimer formation would be minimized. For the same reason, the complementary single-stranded overhangs were also positioned at maximum distance from the dimer. This could have been accomplished in one of two ways. Either restriction enzymes could have be chosen such that one would generate a 3' overhang and the other a 5' overhang. The oligonucleotide complementary to the lesion-containing 11-mer would then contain both single-stranded ends (and would typically, therefore, be a 19-mer). Alternatively, if restriction enzymes were used which both generated either 3' or 5' single-stranded ends, a second oligonucleotide, carrying one of the two complementary single-stranded ends, could be hybridized, alongside the lesion-containing oligomer, to the opposite strand, which carried the second single-stranded end. Although the latter alternative requires one additional ligation step, this was the method used in this study because of the initial choice of restriction enzymes.

Isolation of pGEM-7Zf(-) vector sequences. After insertion of oligonucleotides into the multiple cloning site of pGEM-7Zf(-), the resultant substrate may contain a single cyclobutane dimer (or the modified version). It is necessary to produce large amount of this specific substrate by directly ligating the relevant sequences. Conventional means of achieving large quantities of a specific plasmid (after inserting a gene of interest) or of selecting for a particular insertion, are not applicable to this study as amplification or selection of the vector within *E. coli* results in repair and/or dilution of DNA lesions. For these reasons alone, it was important to ascertain that the only molecules capable of ligating in the final ligation step, to generate a covalently closed circular molecule, would be the three oligonucleotides [AGA-4, AGA-6 (or lesion-containing versions of this oligomer), and AGA-7] to the 2917-bp *Nsi*I/*Sph*I vector fragment. To guarantee this result, the pGEM vector was hydrolysed with two different restriction enzymes, generating a 2917- and a 83-bp fragment. Figure 34 demonstrates the ability of both enzymes alone, or in combination, to cut the plasmid in *Nsi*I buffer. The linear molecule visible after digestion with both enzymes was detectably shorter than that produced by either restriction enzyme alone. (The 83 bp-fragment was not visible on this gel since, in the time allotted, it did not remain in the gel.) Each of the fragments generated by double-digestion cannot re-ligate on itself, as the ends are not complementary.

The two fragments were subsequently separated by gel filtration chromatography (see Table XXII). The initial OD_{260}-detected peak contained the 2917-bp fragment as ascertained not only by the higher absorbance of this peak relative to the later eluting material, but additionally by its migration relative to linear pGEM on an agarose gel (data not shown). Ligation reactions using the 2917-bp sequence alone confirmed that this molecule could not re-ligate to form a covalently closed circle (see Figure 35).

Production and isolation of cyclobutane dimer-containing oligomer AGA-6. Previous studies have utilized acetophenone to increase the proportion of molecules containing a cyclobutane dimer (Ben-Hur *et al.*, 1967; Lamola and Yamane, 1967; Rahn and Landry, 1971; Banerjee *et al.*, 1988). However, this method was not successfully adopted in the work presented here. Although the combination of 10 mM acetophenone and 100 kJ/m^2 of 313-nm light increased the dimer yield, this protocol consistently caused a breakdown of the 11-mer, a not unprecedented result (Rahn *et al.*, 1974), and therefore was not a useful procedure in our hands. Instead, AGA-6 was irradiated with 10 kJ/m^2 254-nm light, inducing a cyclobutane dimer in approximately 10% of the oligomers (see Table XXIII). This was deemed sufficient to ensure adequate quantities of the dimer-containing oligomer.

Purification of T^T-AGA-6 from its parent molecule was carried out using a capped C_{18} reverse-phase column. The separation, achieved with this system, of the molecules produced by irradiation of AGA-6 is illustrated in Figure 36. AGA-6 eluted at fraction 53 under the gradient conditions described in Experimental Procedures. The 11-mer containing a cyclobutane dimer eluted at fraction 37. There was a small peak at fraction 43 presumably associated with (6-4) photoproduct formation (Banerjee *et al.*, 1988). The identity of the cyclobutane dimer-containing peak was confirmed by purifying this material and subsequently monomerizing the cyclobutyl bridge. A small aliquot was either treated with photolyase or exposed to a photochemically reversing fluence of 254-nm light (see Figure 37). Both of these procedures regenerated the parent molecule, supporting the deduction that the purified peak comprised cyclobutane-dimer containing molecules.

As AGA-6 was obtained in an unphosphorylated form, both it, and its T^T-containing derivative, were kinased prior to ligation into the pGEM vector. As any remaining unphosphorylated molecules would reduce the overall efficiency of ligation in the formation of a CCC molecule, those molecules with a 5'-phosphate successfully attached were purified by reverse-phase HPLC. Separation achieved in the case of T^T-containing AGA-6 and its phosphorylated derivative is illustrated in Figure 38. The phosphorylated species peak could easily be identified by [^{32}P] end-labelling a small population of molecules.

Human IDP treatment of T^T-containing AGA-6. Since the detailed chemical structure of a modified dimer has yet to be determined (i.e., whether it is left with a 3', a 5', or no internal phosphate), the most meaningful way to generate a cyclobutane dimer with a severed interpyrimidine linkage was reasoned to be treatment of the T^T-containing oligonucleotide with the human liver IDP which had been purified in our laboratory. Using conditions which were known to cause severage of the intradimer phosphodiester bond of cyclobutane dimers present in either irradiated plasmid DNA or in UV-treated poly(dA)·poly(dT), all indications were that this enzyme had no activity on a single-stranded 11 nucleotide oligomer, presumably due to its size. Efforts to detect IDP-altered molecules initially assumed that reverse-phase HPLC would detect a change in hydrophobicity after the intradimer phosphodiester bond was cleaved. This turned out not to be the case (compare Figure 39, panels A and B). Therefore the IDP treated material was subjected to photoreversal. Cyclobutyl ring monomerization would generate two smaller single-stranded oligonucleotides (a 5-mer and 6-mer) if the interthymidine phosphodiester bond had actually been severed. Using authentic markers (AGA-9 and AGA-8), these expected species could not be detected (see Figure 39C). These experiments were repeated using polyacrylamide gel electrophoresis (PAGE) in case the HPLC methodology employed had not allowed detection of a modified species. PAGE permits assessment of only oligonucleotide length, irrespective of sequence and, presumably, dimer modification. This procedure would then allow accurate measurement of the size of the molecules generated under the various reaction conditions. This, too, failed to reveal any activity of IDP on the dimer-containing oligonucleotide (see Figure 40). Attempts were then made to modify the oligomer, ID-pGEM, with IDP in the presence of the complementary strand (AGA-7). However, conditions allowing hybridization of the short AGA-6 with AGA-7 were not conducive to IDP activity (i.e. high salt concentration and low temperature). Experiments were also repeated after prebinding of photolyase to ID-pGEM in the dark. This strategy was based on the observation that the UvrABC complex has increased activity towards incising cyclobutane dimers in the presence of prebound photolyase (Sancar *et al.*, 1984). These experiments also failed to reveal any activity of IDP on this cyclobutane dimer-containing 11-mer.

Further experimentation indicated a lack of activity of human IDP on single-stranded and double-stranded oligomers of at least less than 25 nucleotides or base pairs in length (data not shown).

Formation of a modified cyclobutane dimer-containing oligonucleotide by the photoligation of AGA-9 and AGA-8. The HPLC markers produced to first detect and to later purify IDP activity from human liver, were generated by photoligation of adjacent unlinked thymidines arising by hybridizing $(dT)_{10}$ to poly(dA) (Liuzzi and Paterson, in press). Photoligation, therefore, seemed a warranted means of generating modified dimer-containing oligonucleotides. For this purpose, two oligonucleotides (AGA-9 and AGA-8), which together comprise the sequence of AGA-6, were hybridized to the complementary strand, and subsequently exposed to a large dose of 254-nm light. HPLC chromatography of the resulting species revealed a novel peak (see Figure 41) representing approximately 2% of the total radioactivity which was present in the original reaction mixture in the form of $[^{32}P]$-AGA-8 (see Table XXIII). This was presumed to be associated with the photoligated oligomers. The retention position of AGA-9 on the HPLC chromatogram depicted in Figure 41 was determined by UV absorbance. It was added to the reaction mixture just prior to injection onto the HPLC. Both AGA-8 and the photoligated 11-mer could be detected by the radioactive profile, as the original hybridization/photoligation reaction had been supplemented with a small amount of $[^{32}P]$-AGA-8. To confirm that this new peak was in fact a photoligated 11-mer, it was purified and subsequently exposed to a photochemically reversing fluence of 254-nm light. Such treatment regenerated AGA-8 (see Figure 42), confirming the anticipated structure of this compound.

As was the case for AGA-6 and its T^T-containing derivative, the photoligated oligonucleotide, AGA-9<>AGA-8, also required the addition of a 5'-phosphate prior to ligation into the pGEM sequence. Phosphorylated molecules were isolated from the non-phosphorylated molecules by HPLC reverse-phase chromatography (see Figure 43). Detection of the phosphorylated derivative was achieved using $[^{32}P]$-5'P-AGA-9<>AGA-8 which had been generated by photoligating $[\gamma\text{-}^{32}P]$ ATP-kinased AGA-9 to AGA-8.

Ligation of the various oligomers into the multiple cloning site of pGEM-7Zf(-) and subsequent purification. Either AGA-6, T^T-containing AGA-6, or AGA-9<>AGA-8, along with AGA-4 and AGA-7, were ligated onto the 2917-bp *Nsi*I-*Sph*I pGEM7Zf(-) fragment. Efficient ligation required a 10 molar excess of oligonucleotides over the vector fragment. In addition, it was critical to allow the molecules to cool slowly from 65°C to 14°C before adding ligase. This presumably ensured correct base-pairing of the oligonucleotides to each other and to the complementary ends of the vector sequence. This modification to the original protocol, alone, increased the yield of final substrate approximately 50-fold (data not shown). As the vector sequence could not ligate on itself to generate a covalently closed circle, the only way such a molecule could be produced was by the ligation of the oligonucleotides to the vector sequence. Since the hybridized oligonucleotides also had non-complementary ends (see Figure 44), only one set of oligonucleotides could be inserted into the vector. After ligation had been completed to form the desired CCC molecule, it would lack supercoiling and would therefore migrate on an agarose gel to the same position as open circle plasmid which, itself, was an undesired byproduct of the reaction and which migrated very close to other unwanted species including linear vector, linear vector with oligonucleotides ligated to one end, and linear concatamers of vector sequence adjoined by oligonucleotides. Therefore, isolation of the desired species was achieved by agarose gel electrophoresis in the presence of ethidium bromide. Ethidium bromide induces positive supercoiling of the covalently closed molecule (Byrne *et al.*, 1964), thus enabling it to migrate well ahead of the other vector-containing species present in the reaction mixture (see Figure 45). The unincorporated oligonucleotides were sufficiently small that they were eluted from the front of the gel. Figure 45 illustrates that the different oligonucleotides (AGA-6, T^T-containing AGA-6, and AGA-9<>AGA-8) ligated to form a CCC molecule with approximately equal efficiency. This was confirmed by a densitometer scanning (Table XXIV) of the gel shown in Figure 45. This information was also used to ascertain the quantity of each substrate produced (see also Table XXV) since a known amount of pGEM vector had been loaded in the first lane. Figure 46 depicts a large scale ligation reaction of ID-pGEM.

Once separation by agarose gel electrophoresis was attained, purification was accomplished by cutting out the relevant gel slice, electroeluting and cleaning the DNA by phenol/chloroform extraction, and finally ensuring the removal of gel contaminants by purification in a Centricon-10. The resultant T^T-containing substrate is depicted in Figure 47. Figure 48 details sequences relevant to *in vitro* replication and transcription analyses.

Assessment of an active DenV preparation. The specific activity of three independent preparations of DenV was ascertain prior to conducting experiments with the lesion-containing constructs (see Figure 49). For this purpose, CCC pGEM was irradiated with a fluence of 254-nm light sufficient to induce, on average, 72 cyclobutane dimers per molecule. An enzyme preparation, capable of converting irradiated CCC DNA to an open circle configuration without effect on unirradiated CCC DNA, was established. One unit of DenV is defined as the amount of enzyme required to nick 1 fmol of 5.5 x 10^6 Da CCC DNA (which contains an average of 2 cyclobutane dimers/molecule) in 15 min at 37°C (Seawell *et al.*, 1981). Therefore, the amount of DenV required to nick 20% of the CCC DNA in this reaction is approximately 36 units. It required 0.5 µl of the selected DenV preparation to nick 20% of the UV-irradiated CCC DNA (data not shown). This DenV preparation, therefore, had a concentration of ~70 units/µl.

The action of the UV endonuclease on cyclobutane dimers with or without a severed intradimer phosphodiester bond. Prior to experiments utilizing the three substrates (m-pGEM, ID-pGEM, and MD-pGEM), they were first restricted with *Pvu*II and subsequently [^{32}P] end-labelled, generating two double-stranded radioactively-labelled fragments (2567- and 373-bp in length). If DenV were to make a single-stranded cut at the cyclobutane dimer (which was contained within the 373-bp fragment of ID-pGEM), a 142-nt single-stranded end-labelled fragment would be detected on a polyacrylamide gel under denaturing conditions. (The remainder of this strand, a 231-nt fragment, had no label and was therefore not detected.) The outcome of such an experiment is illustrated in the autoradiogram depicted in Figure 50. Results from similar experiments using *Pvu*II restricted / end-labelled m-pGEM and MD-pGEM, with and without DenV treatment, are shown in the same diagram. In addition, this figure includes the results of an experiment in which ID-pGEM was hydrolysed with IDP prior to DenV treatment. Data obtained from a densitometer scanning of this autoradiogram are summarized in Table XXVI. As polyacrylamide gels were run under conditions which allowed separation of the 373- and 142-nt fragments, the 2567-nt sequence could not enter the gel and was instead retained in the well. Consequently, densitometry was performed only on the smaller fragments.

As would be predicted, in lane 1, containing *Pvu*II-cut m-pGEM, only a 373-nt fragment was seen on the gel. Lanes 5 and 9 demonstrate that incubation of this substrate with T4 DenV protein did not alter this result. Thus, the DenV preparation used was not contaminated by nonspecific nucleases. Lanes 2 and 3 show untreated *Pvu*II-cut MD-pGEM and ID-pGEM, respectively. Whereas the 373-nt fragment is by far the major species in lanes 2 and 3, a 142-nt band is significant, even without DenV treatment, in lane 2. As is illustrated by lanes 7 and 11 of Figure 50 and in Table XXVI, DenV treatment of *Pvu*II-cut ID-pGEM generated a 142-nt band, representing 36.6% and 34.6% of the two smaller (142- and 373-nt) bands, respectively. This was the maximum intensity which could be realized in this lower band, even with an increased amount (80-fold excess) of the dimer-recognizing enzyme. DenV treatment of MD-pGEM (lane 6), on the other hand, did not alter the intensity of the 142-nt band (18.4% as compared to 16.9%). Although pretreatment of ID-pGEM with human liver IDP still allowed DenV to increase the proportion of material associated with the 142-nt fragment (from 0% up to 12.5%; lanes 3 and 10, respectively), this occurred to a lesser extent than when this substrate has not been predigested with IDP.

Calculation of the activity of the DenV preparation (i.e., 70 units/μl) indicated that the quantity of DenV used in the experiments illustrated in Figure 50, was approximately twice that needed to produce single-stranded nick at every cyclobutane dimer present. As alluded to above, increasing the amount of DenV protein present in any of the reactions (an 80-fold excess) did not significantly change the results. At this higher enzyme concentration, m-pGEM remained unaltered, and the intensity of the 142-nt band did not increase beyond 38% for DenV-digested ID-pGEM and 18.4% for MD-pGEM.

DNA synthesis by *E. coli* DNA polymerase I on a substrate containing a cyclobutane dimer with or without a severed intradimer phosphodiester bond. Prior to *in vitro* replication assays, each substrate was linearized with a restriction enzyme (*Apa*I, *Acy*I or *Pvu*II) and annealed to the reverse sequencing primer. To date, only one experiment per restricted substrate has been performed, and therefore ideal conditions have not yet been defined as yet. However, a preliminary experiment, using *E. coli* DNA polymerase I to synthesize DNA from the reverse sequencing primer of *Apa*I restricted substrates, is illustrated in Figure 51. The length of a newly replicated molecule, starting from the reverse sequencing primer, synthesized in the presence of labelled TTP, and terminating at the *Apa*I site, is 98 nt. On the other hand, if the polymerase is blocked by the lesion, this labelled DNA molecule would be only 80 nucleotides long. As shown in Figure 51, synthesis from the annealed primer on *Apa*I-cut m-pGEM generated two molecules, one migrating slightly ahead of the 104-nt marker, and the other migrating slightly behind. In the case of the other two substrates, ID-pGEM and MD-pGEM, the labelled DNA synthesized by *E. coli* DNA polymerase I was again of two discrete sizes, but now migrating at the location of the 80-nt marker. These bands were relatively faint and were the only species detected. This result was consistent for the different restriction enzymes used.

Transcription by SP6 RNA polymerase on a substrate containing an intact or modified cyclobutane dimer. After linearizing the various substrates, transcription was initiated from the SP6 start site in the presence of radioactively labelled UTP. Preliminary experiments, utilizing *Apa*I-cut substrates, are illustrated in Figure 52. A run-off transcript, beginning from the SP6 start site, would be 49 nt in length. One whose progression is impaired by a lesion would be 30-33 nt long. In the polyacrylamide gel depicted in Figure 52, transcripts generated in each reaction have been electrophoresed alongside DNA markers. As RNA will migrate through this matrix at a slower rate than DNA due to inherent secondary structure (Maniatis *et al.*, 1982), the transcripts migrating at any given position are shorter than indicated by the nearby DNA markers, but this effect is only barely noticeable for short RNA molecules like those synthesized in these experiments. Lane 1 displays the transcripts produced by the SP6 polymerase using *Apa*I-cut m-pGEM DNA as a substrate. The major transcript migrated roughly at the position of the 51-nt DNA marker. (Other minor bands did not migrate in the region of the gel depicted in Figure 52.) Lane 2 contains transcripts synthesized from ID-pGEM. There were two bands present, the upper corresponding to the major band seen in lane 1. The lower band migrated to a position approximately half way between the 21- and 51-nt DNA markers. Transcripts from MD-pGEM are displayed in lane 3. The major band was the same as that seen in lane 1. A minor band migrated slightly slower than the lower band observed in lane 2. Thus, in the case of transcription initiated from the SP6 promoter of m-pGEM, the major transcript appears to correspond in length to the expected run-off transcript of 49 nt. On the other hand, the major transcript synthesized from ID-pGEM appears to correspond to that expected (33 nt) if progression of the RNA polymerase was impaired by the cyclobutane dimer. There were, however, some presumed full-length run-off transcripts from this reaction. When MD-pGEM served as the template for transcription, the major transcript corresponded in length to the presumed full-length run-off transcript. However, a minor transcript, larger than that synthesized from ID-pGEM, was also evident. Results consistent with these were obtained regardless of the restriction enzyme used to linearize the substrates (data not shown).

DISCUSSION

Both prokaryotic and eukaryotic cells possess an intradimer phosphodiesterase activity which severs the interpyrimidine phosphodiester linkage of a cyclobutane dimer. Modified dimer sites appear in the oligonucleotides excised during repair of UV-induced DNA damage in normal human fibroblasts (Weinfeld *et al.*, 1986; this study), CHO cells (this study), and wild-type *E. coli* (this study). Furthermore, these altered sites accumulate during post-UV incubation in the genomic DNA of human (Paterson *et al.*, 1984; Paterson *et al.*, 1987; this study), CHO (Pirsel *et al.*, 1989), and *E. coli* (this study) repair-deficient cell strains. Substrates have now been constructed which, for the first time, allow direct assessment of the action of various polymerases and repair enzymes on a cyclobutane dimer with a cleaved intradimer phosphodiester linkage. These double-stranded covalently closed circular molecules employ a pGEM vector into which oligonucleotides have been inserted. The inserted 11-mer has (i) no lesion, (ii) a cyclobutane dimer at the unique dipyrimidine site, (iii) or a modified dimer at this identical position. The insertion position is within the multiple cloning site of the plasmid, producing a substrate which is readily amenable for the study of *in vitro* replication and transcription as necessary control sequences are in close proximity. Although these substrates may be more complex than needed for basic analysis of the activity and affinity of repair enzymes such as T4 DenV, they are immediately amenable to these experiments and, furthermore, easily manipulated to a more *in vivo*-like state (such as supercoiling), if required. These novel constructs also have other *in vitro*, as well as *in vivo* applications, which will be discussed in detail in the final chapter of this dissertation.

In generating large quantities of the different covalently closed circular substrates, many non-trivial factors needed to be considered and controlled. As two of the three final substrates contain DNA lesions, large quantities could not be propagated by conventional amplification schemes. Instead, they needed to be produced by direct ligation of specific oligonucleotides into vector sequences. The lack of ability to select for an end-product required that the only covalently closed circular molecules able to form were the desired constructs. Thus, vector sequences could not be allowed to ligate on themselves, nor could multiple copies of the oligonucleotides be permitted to insert into the vector.

The strategy followed to circumvent any foreseeable problems has been outlined in Experimental Procedures. To recapitulate, this procedure involved cutting the pGEM-7Zf(-) vector with two different restriction enzymes to generate non-complementary ends, and subsequently isolating the desired longer fragment by gel filtration. The purchased oligonucleotides contained the identical non-complementary ends. Next, to ensure the ligation of a pure population of a cyclobutane dimer-containing 11-mer into this vector sequence (in the construction of ID-pGEM), the T^T-containing oligonucleotides were isolated from their parent molecules by reverse-phase HPLC chromatography following irradiation of the population with 10 kJ/m^2 of 254-nm light. The single most important parameter, discerned in this study, which enabled separation of these two species, was the use of a capped reverse-phase column.

The matrix of the reverse-phase column used for this purpose is silica. C_{18} functional groups are attached to the silica matrix, and the resulting stationary phase serves to elicit the hydrophobic interactions necessary to effect separation of various molecules. If the column has not been end-capped, non-reacted silica groups remain and, as a result, separation is no longer based solely on hydrophobic interactions, but additionally on silanophilic associations. Hence, capping of the column ties up the previously nonreacted silica, thus eliminating silanophilic interactions. This was essential to enable separation of the cyclobutane dimer-containing AGA-6 from its progenitor molecule. Presumably, the silanophilic interactions are viewed by the two species in an equal way. If these outweigh the differing hydrophobic associations, separation is nullified unless the silanophilic interaction are removed.

Irradiated AGA-6 comprised approximately 10% T^T-containing molecules (see Figure 38). The authenticity of this species was confirmed by subsequent treatment with photolyase or by photochemical reversal (see Figure 37). These processes, which monomerize only a cyclobutane dimer (Rahn and L.C. Landry, 1971; Mitchell and Clarkson, 1984), regenerated the parent species.

In a similar way, in generating MD-pGEM, the insertion of a pure population of oligomers with a modified cyclobutane dimer was required. Although the original intent was to generate these molecules by intradimer phosphodiesterase treatment of the T^T-containing oligomers, this proved to be unproductive. Experiments such as those illustrated in Figures 39 and 40 divulged the inactivity of this enzyme on a single-stranded oligonucleotide of ≤11 nucleotides in length. [Other experiments (not shown here) illustrated inactivity on single-stranded or double-stranded DNA of ≤25 nucleotides or base pairs.] As IDP cleaves the phosphodiester bond between two dimerized pyrimidines, it was anticipated that this reaction would expose a terminal phosphate, thus altering the retention time of the resultant molecule on a reverse-phase column. This did not turn out to be the case (compare Figure 39, panels A and B). As it could not be assumed that separation of the two expected species was possible by the chromatography conditions employed, T^T-containing AGA-6, which had been pre-treated with IDP, was further subjected to photochemical reversal. If the intradimer phosphodiester bond had been severed by hydrolysis with IDP, subsequent removal of the lesion would release two smaller oligonucleotides, a 5-mer and a 6-mer. Authentic markers for these two smaller species had been made available, and thus the outcome of the reaction and subsequent HPLC analysis could be evaluated with certainty. As illustrated in Figure 39C, this series of reactions did not generate the two smaller oligonucleotides, but instead, only the non-lesion-containing 11-mer. This substantiated that IDP did not cleave the intradimer phosphodiester bond of the cyclobutane dimer present in this short single-stranded oligonucleotide, although hydrolysis conditions were identical to those known to effect this reaction on a cyclobutane dimer contained within a large double-stranded DNA molecule. Analysis was repeated and the conclusion confirmed using polyacrylamide gel electrophoresis (see Figure 40). IDP treatment did not alter the mobility of T^T-containing AGA-6, as would be expected since it severs the phosphate backbone but does not decrease the number of nucleotides. However, photochemical reversal also caused no change in the size of IDP treated T^T-containing AGA-6. Therefore, as this process is expected to release a 5-mer and 6-mer after cleavage of the intradimer phosphodiester bond, it could again be surmised that IDP did not operate on these molecules.

To circumvent the problem of the inactivity of IDP towards T^T-containing AGA-6, a modified dimer-containing oligonucleotide was constructed by photoligating two shorter oligomers (Lewis and Hanawalt, 1982; Liuzzi and Paterson, in press). These two smaller oligonucleotides, AGA-9 and AGA-8, together comprise the sequence of AGA-6 (see Figure 33). By choosing this route, it is assumed that the structure of an authentic modified dimer is essentially the same as that generated by this process. Although the marker molecule, d-TpT<>dT, used to detect and purify human liver IDP activity was devised by photoligation (Liuzzi and Paterson, in press), the position of the terminal phosphate produced by the cleavage, if it exists at all, is not known. This phosphate could be attached to either the 5' or 3' nucleotide of the cyclobutane dimer. However, the assumption was made that by constructing a molecule with this phosphate on the 5' dimerized pyrimidine, a reasonable facsimile of the modified dimer would be generated. The action of various cellular processes on encountering such a lesion could later be compared to the genuine modified cyclobutane dimer generated by IDP treatment of the double-stranded circular dimer-containing substrate, ID-pGEM.

Irradiation (5.5 kJ/m^2) of the two small oligomers, AGA-9 and AGA-8 (after annealing to AGA-7), produced photoligated molecules in the order of 2% of the input AGA-8. This novel species was isolated by reverse-phase HPLC (see Figure 41) and its authenticity verified by photochemical reversal (see Figure 42). In this figure, as AGA-8 was the only [^{32}P] end-labelled input molecule, it is the only visible parent species regenerated by monomerization of the cyclobutane dimer which bridges AGA-9 to AGA-8.

As at least 5 - 50 fmol of substrate are required for any given polymerase or repair enzyme reaction, a significant quantity of each had to be produced. Aiming at a final yield of 1-2 pmol per construct, ligation reactions were set up utilizing 16 pmol of vector sequence and a 10-fold excess of each oligonucleotide to be inserted (see Table XXV). The optimal overabundance of oligomers to vector had been determined empirically, and although 100-fold gave a slightly higher yield of product, 10-fold gave a balance between sufficient production and conservation of the valuable oligomers. The inability of the 2917-bp pGEM fragment to re-ligate, generating a covalently closed circular molecule, was ascertained and, although ligation products were seen (see Figure 35), there were no species produced migrating in the region of interest. As the CCC molecules generated by ligating vector and oligonucleotides would not be supercoiled, the low melt agarose gels used to separate these species from parent molecules and other ligation products, contained sufficient ethidium bromide to induce positive supercoiling in a CCC DNA molecule. This allowed migration of the desired end-product to a location in the gel where it could be easily purified from unwanted material. After removal from the gel by electroelution, the substrates were cleaned by phenol/chloroform extraction and subsequently, on a Centricon-10 concentrator. These latter two procedures were essential to removing contaminants found capable of inhibiting further enzymatic reactions.

The resultant ID-pGEM substrate is diagrammed in Figure 47. An enlargement of the multiple cloning site region, into which the oligonucleotides were inserted, is illustrated in Figure 48. The features of this system are immediately obvious. First of all, this system is easily adapted to study the *in vitro* activity of various repair enzymes on the various substrates. Secondly, authentic replication origins lie in the vicinity (*f1* and *E. coli ori*) of the inserted oligonucleotides. However, the availability of both forward and reverse sequencing primers allow ready *in vitro* replication by any DNA polymerase. Finally, the action of several RNA polymerases can be directly assessed. Transcription can be initiated *in vitro* from the SP6 promoter, placing the lesion on the transcribed strand. Alternately, it can be instated at the T7 promoter. This would position the lesion on the non-transcribed strand. In addition, since the multiple cloning site is within the *lacZ* gene, in the presence of c-AMP receptor protein, *in vitro* transcription can be initiated from this promoter with *E. coli* RNA polymerase.

Preferential repair of cyclobutane dimers from transcriptionally active sequences has been described in several systems (Bohr *et al.*, 1985; Mellon *et al.*, 1986; Mellon *et al.*, 1987; Mellon and Hanawalt, 1989; Smith and Mellon, 1989). Its detection is dependent on the ability of DenV to induce a single-stranded break at the site of a cyclobutane dimer (see Figure 31). Repair is assumed to be reflected by the loss of these UV endonuclease-sensitive sites. The action of DenV on the lesions contained within the various substrates questions the use of this UV endonuclease to monitor the removal of cyclobutane dimers from genomic DNA and the interpretation of the results obtained from such studies.

In contemplating a cellular function for intradimer phosphodiesterase, one hypothesis has envisioned intradimer phosphodiester cleavage as a means of alleviating the structural distortion which interrupts base-pairing. This would presumably, in turn, allow the replication and/or transcription machinery to bypass this otherwise polymerase-blocking lesion (Caillet-Fauquet *et al.*, 1977; Villani, G., *et al.* 1978; Moore *et al.*, 1981; Vos and Rommelaere, 1982; Griffiths and Ling, 1989; Selby and Sancar, 1990). The first test of this speculation was executed in our laboratory on normal and XP group D human fibroblasts, and employed the same procedure used to uncover preferential repair, with one critical variation. In addition to treating the restricted DNA with DenV (see Figure 31), an aliquot was subjected to photoenzymatic reversal. The results of this study indicated that at least a portion of the cyclobutane dimers surmised to be preferentially repaired from transcriptionally active sequences in human cells, were actually modified rather than eliminated. Furthermore, the modified cyclobutane dimer sites found previously in post-UV incubated XP-D cells (Paterson *et al.*, 1984; Paterson *et al.*, 1987), were shown in this study to be frequently associated with transcriptionally active sequences. Thus, cyclobutane dimer modification is implicated in facilitating transcription on a damaged template.

To address this premise further and challenge the use of the T4 UV endonuclease in preferential repair studies, the ability of DenV to act on the substrates described in this chapter (m-pGEM, ID-pGEM, MD-pGEM, and ID-pGEM pretreated with IDP) was tested. This investigation demonstrated several things. First of all, as is clear from Figure 50 and the densitometric results presented in Table XXVI, the MD-pGEM preparation was contaminated with a population of molecules with a single-stranded nick at the location of the cyclobutane dimer. [^{32}P] end-labelling of the *Pvu*II restricted substrate, and subsequent denaturing polyacrylamide gel electrophoresis, revealed not only the expected 373-nt fragment, but also a 142-nt band. Subsequent electrophoresis of this material on a non-denaturing gel confirmed that this nick was, in fact, a single-stranded nick and not a double-stranded break (data not shown). This contamination was substantial, with 16.9% of the radioactivity present in the two smaller bands being associated with the smaller 142-nt fragment. This corresponds to 33.8% of the substrate molecules. This smaller fragment was most likely present as a result of monomerization of the cyclobutane dimer which bridges AGA-9 and AGA-8. As the photoligated molecules were purified by HPLC, and as they migrate to a position quite separate from the parent compounds, it is unlikely that contamination of nondimerized material occurred at this stage. It is much more likely that exposure to short-wave ultraviolet light (10-20 min in total), during manipulations associated with electroelution, has induced photochemical reversal in a portion of the molecules. However, this did not abolish the usefulness of the MD-pGEM substrate to test the ability of DenV to induce further single-stranded nicked at the site of the modified cyclobutane dimer. In fact, this information made interpretation of *in vitro* transcription results possible. It is, however, something that need be corrected before proceeding further with these studies (see Chapter 9).

As may be expected from the loss of dimers in MD-pGEM, the ID-pGEM preparation also appeared to include nondimer-containing molecules. This was not obvious until extensive studies had been performed with increasing concentrations of DenV. Sufficient quantities of DenV will induce a single-stranded break at every cyclobutane dimer. As all of the molecules present in the ID-pGEM preparation supposedly had one cyclobutane dimer, generous quantities of DenV were expected to reveal an equal 373-nt band (the nondimer-containing strand) and 142-nt band (from having incised at the cyclobutane dimer) on the denaturing polyacrylamide gel. However, the ratio of 142-nt fragment to 373-nt fragment could not be raised past ~2:3, even with increasing the quantity of DenV 40-fold (i.e., to an 80-fold excess over that required to incise every cyclobutane dimer present). This implies that ~24% of the purified ID-pGEM population has also lost the cyclobutane dimer.

Although it was now clear that the dimer-containing substrates were not pure, they were still useful in revealing the activity of DenV on a modified versus intact cyclobutane dimer. As mentioned previously, treatment of ID-pGEM with DenV generated the expected 142-nt band. However, the same concentration of enzyme did not increase the density of the corresponding band in the MD-pGEM + DenV reaction. Even when the amount of DenV was raised 40-fold in the MD-pGEM reaction, there was no significant change in the proportion of end-labelled molecules migrating at this position (see Table XXVI). This demonstrates that DenV is inactive on, at least, the modified dimer generated by photoligation.

To test the ability of DenV to incise an authentic modified cyclobutane dimer, ID-pGEM was hydrolysed with human liver IDP prior to DenV treatment. As shown in Figure 50 and summarized in Table XXVI, when IDP treatment preceded DenV hydrolysis, the intensity of the 142-nt band was reduced (34.6% to 12.5%). This implies that DenV has, at least, reduced activity on an authentic modified dimer. However, if IDP modification renders a cyclobutane dimer completely refractory to DenV, then, under the experimental conditions employed, IDP had modified 68% of the cyclobutane dimers present in the sample, to leave 25% of the molecules still containing an intact cyclobutane dimer, 53% with a modified lesion, plus the preexisting 24% which lacked a dimer. To verify this proposed explanation, reaction conditions need to be established under which subsequent DenV treatment of IDP-altered, PvuII-cut ID-pGEM does not generate a 142-nt band. However, as increased DenV concentration could not further decrease the intensity of the 142-nt band on IDP pretreated substrate, the results of this experiment suggest that IDP hydrolysis of a cyclobutane dimer generated a structure which was impervious to DenV. This investigation also suggests that the structure of the modified dimer (generated through photoligation) is similar, if not identical, to the authentic IDP-altered lesion. This warrants the use of MD-pGEM in other studies.

This inability of DenV to recognize a cyclobutane dimer with a severed intradimer phosphodiester bond necessitates reevaluation of the data which has been accumulated on preferential repair of transcriptionally active genes. It is impossible to say at this point whether or not cyclobutane dimer modification accounts for all preferential repair. Furthermore, it will be important to assess the action of IDP on other bulky lesions, such as aflatoxin B_1 and psoralen photoadducts, known to display preferential repair (Leadon and Hanawalt, 1986; Vos and Hanawalt, 1987).

Only preliminary experiments have been completed for the proposed *in vitro* replication and transcription studies. Because it has not been trivial to establish appropriate reaction conditions, there is still much work to be done in this area. However, the results obtained to date suggest that cyclobutane dimer modification allows transcription bypass (at least in the case of SP6 RNA polymerase) but has no effect on the progression of the replication complex (at least for *E. coli* DNA polymerase I).

As illustrated in Figure 51, *in vitro* replication on the *Apa*I-cut m-pGEM generated the expected run-off sequence of approximately 98 nucleotides (the distance from where the reverse sequencing primer anneals to the *Apa*I site, and including the length of the primer). The doublet seen at this position may reflect some variation in the size of the primer at its 5' end as it was seen also when either of the other two substrates were used as template DNA. This has not been verified. When either ID-pGEM or MD-pGEM was used as a template for replication, the labelled product was significantly shorter. In both instances, a doublet which migrated alongside the 81-nt DNA marker, was the only species present. This is the expected length of a newly replicated DNA strand which begins at, and includes, the reverse sequence primer, but cannot proceed past the lesion at position 32-33. This is predicted for the cyclobutane dimer-containing substrate, as it has been demonstrated previously that a cyclobutane dimer acts as an absolute block to the progression of the replication complex (Caillet-Fauquet *et al.*, 1977; Villani, G., *et al.* 1978; Moore *et al.*, 1981; Griffiths and Ling, 1989). *E. coli* DNA polymerase I was also unable to pass the modified cyclobutane dimer. It is, therefore, unlikely that IDP functions in replication bypass for at least this polymerase. However, as the major function of DNA polymerase I is in repair synthesis, it is unlikely that it often encounters this dilemma. These results may, therefore, not reflect what would be seen using a major DNA replication complex such as *E. coli* DNA polymerase III. Furthermore, cells seem to possess some mechanism to overcome these barriers to DNA replication (Vos and Rommelaere, 1982). Although the three-dimensional configuration changes undergone after intradimer phosphodiester cleavage are unknown, this result would imply that base-pairing distortion is not corrected by this modification.

The presence of contaminating non-dimer containing ID-pGEM would be expected to produce some full length run-off molecules. However, as the bands on this gel are quite faint, it is quite possible that only the major species in each reaction are detectable. From the DenV experiments, the nondimer-containing molecules represent about 24% of the population, and given the barely discernable bands with a length of approximately 80 nt, it is conceivable that a 3-fold less prevalent band would not be detected.

A preliminary experiment using the various substrates as templates for SP6 transcription, are illustrated in Figure 52. All substrates have been linearized with *Apa*I prior to *in vitro* transcription reactions. When m-pGEM was used as a template molecule, the major transcript migrated slightly above the 51-nt DNA marker. It should be remembered that RNA migrates more slowly through the gel matrix than DNA due to its inherent secondary structure. This major band, therefore, likely corresponds to the 49-nt run-off transcript expected when synthesis is initiated at the SP6 promoter and is terminated by the *Apa*I-generated free end. There were also minor species present, which represented only a small percentage of the total transcripts. They likely reflect premature terminations.

For the case employing ID-pGEM as the template molecule, the major band seen after denaturing polyacrylamide gel electrophoresis migrated approximately midway between the 51- and 21-nt DNA markers. It, most likely, represents the transcript which terminates at the site of the cyclobutane dimer and should be, therefore, approximately 30 nucleotides long. Transcription has been shown previously to be terminated by a cyclobutane dimer (Selby and Sancar, 1990). There was also a band which correlated with the major band generated when m-pGEM was employed as the template. This, undoubtedly, was due to the contamination of nondimer-containing molecules, and represents full length run-off transcripts.

The intriguing result came from *in vitro* transcription across the modified dimer present in MD-pGEM. As seen in Figure 52, denaturing polyacrylamide gel electrophoresis revealed two bands. The major band migrated parallel to that in the m-pGEM reaction and therefore presumably depicts full length run-off transcripts. That this was, by far, the major species present, implies that SP6 RNA polymerase can bypass this modified cyclobutane dimer. As was the case for ID-pGEM, DenV experiments had revealed that the MD-pGEM preparation was contaminated with nondimer-containing molecules. The loss of the cyclobutane dimer which bridges AGA-9 and AGA-8 would generate a single-stranded nick between the nucleotides at positions 32 and 33. Some faster migrating transcripts had, therefore, been anticipated, due to run-off transcription at this break in the DNA. However, in marked contrast to the major band seen when transcription was blocked by the cyclobutane dimer contained within ID-pGEM, this band migrated slightly slower, and therefore represents a slightly larger species. As the exact position of the nick generated by monomerization of the cyclobutane dimer present in MD-pGEM is known, the length of this fragment must be 32 nt in length. It is difficult to predict from these experiments how much shorter the transcripts which emerged from the ID-pGEM reaction were, but they were obviously at least 1 nt shorter, and given the separation in this region of DNA markers of known length, they were more likely in the neighborhood of 2-3 nt shorter. This implies that transcription stops several nucleotides in advance of the dimerized pyrimidines. It is not surprising the polymerase cannot use either dimerized thymidine as a template (yielding a 31-nt transcript), but it appears that the process was interfered with one or two nucleotides preceding this lesion. This could be caused by a region of inadequate base-pairing, provoked by the cyclobutane dimer.

The simplest hypothesis explaining transcription past a modified cyclobutane dimer is that base-pairing is restored. However, the lack of bypass by *E. coli* DNA polymerase I argues against this theory. It is possible, however, that since this replication polymerase normally functions in gap filling during repair, it is blocked by a cyclobutane dimer because it specifically recognizes its structure, as well as being sensitive to the base-pairing distortion. The most indisputable means of assessing whether or not recovery of base-pairing occurs would be to model the change in structural conformation effected by cleavage of the intradimer phosphodiester bond.

It is clear that only the surface has been scratched as far as our understanding of cyclobutane dimer modification is concerned. It appears to be a central part of what has been described as preferential repair, although *in vivo* experiments are necessary for confirmation. It appears to allow at least one RNA polymerase to bypass, although these experiments were performed with an artificially constructed modified lesion. Further work promises to enhance our understanding of this enzyme which now appears to be a key player in circumventing damage induced by ultraviolet light. The various directions currently planned for this work are described briefly in the final chapter of this dissertation.

fractions	OD_{260}	DNA (μg)	fragment
15-16	0.000	0.0	-
17-18	0.067	2.7	
19-20	1.598	65.5	2917 bp
21-22	2.850	116.8	
23-24	1.738	71.3	
25-26	0.791	32.4	
27-28	0.207	8.5	
29-30	0.005	0.2	
31-32	0.000	0.0	
33-34	0.000	0.0	
35-36	0.000	0.0	
37-38	0.000	0.0	
39-40	0.002	0.1	
41-42	0.045	1.8	83 bp
43-44	0.082	3.4	
45-46	0.071	2.9	
47-48	0.006	0.2	
49-50	0.000	0.0	
51-52	0.000	0.0	

TABLE XXII OD_{260} reading and amount of DNA in the various fractions of *Nsi*I/*Sph*I-digested pGEM-7Zf(-) eluted from the Sepharose CL-6B column.

component	amount irradiated	quantity of dimer-containing species isolated	efficiency of reaction
AGA-6	300 nmol	T^T-containing AGA-6 29.1 nmol	9.7%
AGA-9	360 nmol	AGA-9<>AGA-8 2.07 nmol	2.3%
AGA-8	90 nmol		
AGA-7	90 nmol		
	quantity restricted	quantity isolated by gel filtration	% recovery
2917 bp fragment of pGEM-7Zf(-)	1.12 mg	624 µg	55.7%

TABLE XXIII Tabulation of the quantities involved and the efficiency of reactions for the procedures involving (i) isolation of the 2917-bp pGEM-7Zf(-) fragments and (ii) isolation of cyclobutane dimer and modified dimer-containing oligonucleotides.

lane	band	ligation reaction	amount of DNA loaded	densitometric reading	quantity of DNA in band
1	top	uncut pGEM-7Zf(-)	350 ng	0.065	33 ng
	bottom			0.620	317 ng
4	bottom	m-pGEM	1.56 µg	0.274	122 ng
5	bottom	ID-pGEM	1.56 µg	0.209	107 ng
6	bottom	MD-pGEM	1.56 µg	0.211	108 ng

TABLE XXIV Densitometric scan, of the ligation reactions shown in Figure 45, to quantitate amount of resultant substrate formed.

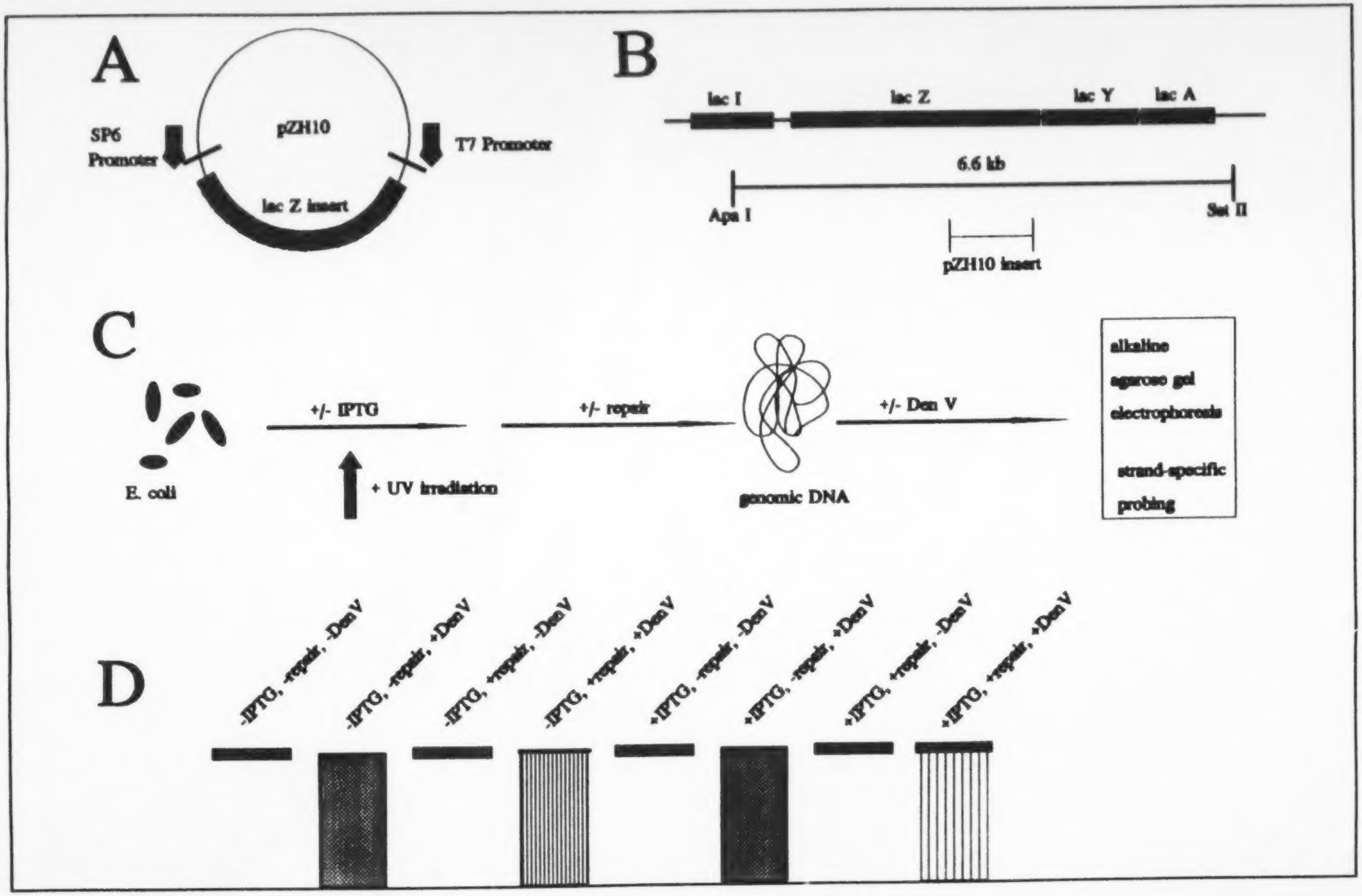
component or substrate	quantity utilized in reaction	relative molar quantity
AGA-6	160 pmol	10
T^T-containing AGA-6	160 pmol	10
AGA-9<>AGA-8	160 pmol	10
AGA-4	160 pmol/reaction	10
AGA-7	160 pmol/reaction	10
2917-bp fragment of pGEM-7Zf(-)	31.2 µg/reaction (16 pmol)	1
	quantity of resultant substrate	efficiency
m-pGEM	3.3 µg	10.6%
ID-pGEM	2.1 µg	6.7%
MD-pGEM	2.2 µg	7.1%

TABLE XXV Quantities of components utilized and efficiency of reaction in ligating oligonucleotides into the multiple cloning site of pGEM-7Zf(-).

lane	substrate	band	fragment	densitometer reading	% of material in band
1	m-pGEM	major	373 bp	2.477	100.0
2	MD-pGEM	upper	373 bp	1.954	83.1
		lower	142 bp	0.396	16.9
3	ID-pGEM	major	373 bp	1.719	100.0
4	markers	-	-	-	-
5	m-pGEM + DenV	major	373 bp	1.977	100.0
6	MD-pGEM + DenV	upper	373 bp	1.566	81.6
		lower	142 bp	0.354	18.4
7	ID-pGEM + DenV	upper	373 bp	1.395	63.4
		lower	142 bp	0.804	36.6
8	markers	-	-	-	-
9	m-pGEM + IDP + DenV	major	373 bp	1.847	100.0
10	ID-pGEM + IDP + DenV	upper	373 bp	1.238	87.5
		lower	142 bp	0.178	12.5
11	ID-pGEM + DenV	upper	373 bp	1.485	65.4
		lower	142 bp	0.786	34.6

TABLE XXVI Representative densitometric scan of relevant bands generated upon DenV hydrolysis of *Pvu*II cut [^{32}P] end-labelled pGEM-derived constructs. DNA bands were detected by polyacrylamide gel electrophoresis as illustrated in Figure 49.

FIGURE 31 Assay system used by Mellon and Hanawalt (1989) to detect repair of cyclobutane dimers in specific regions of the genome (in this example for *E. coli*). **A:** Vector utilized in generation of strand-specific probes for the *lacZ* gene. **B:** The *lac* operon of *E. coli* illustrating the location of (i) the *ApaI/Sst*II restriction fragment monitored in preferential repair studies, and (ii) the pZH10 insert used in **A** to generate strand-specific probes. **C:** *E. coli* cells are grown in the presence or absence of the *lac* operon transcription inducer, IPTG. Cultures are then irradiated with 40 J/m^2 of 254-nm light, and harvested either immediately (to establish the number of cyclobutane dimers induced), or after incubation for increasing periods of time (under the established pre-irradiated induction conditions). Nonreplicated high-molecular-weight DNA is purified from each culture and restricted with *Apa*I and *Sst*II. One-half of each sample is treated with DenV in order to generate a single-strand nick at the site of each cyclobutane dimer. The other half is left untreated. Samples are then electrophoresed on a denaturing gel, and Southern blotted. These blots are hybridized to the transcribed strand-specific radioactively-labelled probe generated from the vector illustrated in **A**. **D:** Results from such an experiment. Lane 1: Cells have been grown under non-inducing conditions (-IPTG) and harvested immediately following irradiation. DNA has not been treated with DenV. The only detectable band corresponds to the 6.6 kb probed region. Lane 2: The source of DNA is the same as for lane 1 but it has been treated with DenV. DenV generates a single-strand break at each dimer cyclobutane dimer. As dimers occur at random positions within this sequence, DenV treatment results in the reduction of a discrete 6.6 kb band. The relative intensity of this band represents the number of 6.6 kb fragments which lack dimers, and is used to calculate the average number of dimers/fragment. Lane 3: DNA from post-UV incubated (10 min) cells grown under non-inducing conditions. Lane 4: DNA is from the same source as in lane 3 but has been treated with DenV. The intensity of the 6.6 kb band is greater than in lane 2, presumably because a portion of the cyclobutane dimers in this non-transcribed sequence have been repaired. Lane 5: Cells have been grown under inducing conditions (+IPTG) and harvested immediately following irradiation. Lane 6: DNA is from the same source as in lane 5 but has been treated with DenV. As there has been no repair, results are the same as lane 2. Lane 7: DNA from post-UV incubated (10 min) cells grown under inducing conditions. Lane 8: DNA is from the same source as lane 8 but has been treated with DenV. The 6.6 kb band has barely been reduced in intensity, presumably because the majority of the dimers in this transcribed sequence have been repaired.


A
SP6 Promoter
pZH10
T7 Promoter
lac Z insert
B
lac I
lac Z
lac Y
lac A
6.6 kb
Apa I
Sst II
pZH10 insert
C
E. coli
+/- IPTG
+ UV irradiation
+/- repair
genomic DNA
+/- Den V
alkaline
agarose gel
electrophoresis
strand-specific
probing
D
-IPTG, -repair, -DenV
-IPTG, -repair, +DenV
-IPTG, +repair, -DenV
-IPTG, +repair, +DenV
+IPTG, -repair, -DenV
+IPTG, -repair, +DenV
+IPTG, +repair, -DenV
+IPTG, +repair, +DenV

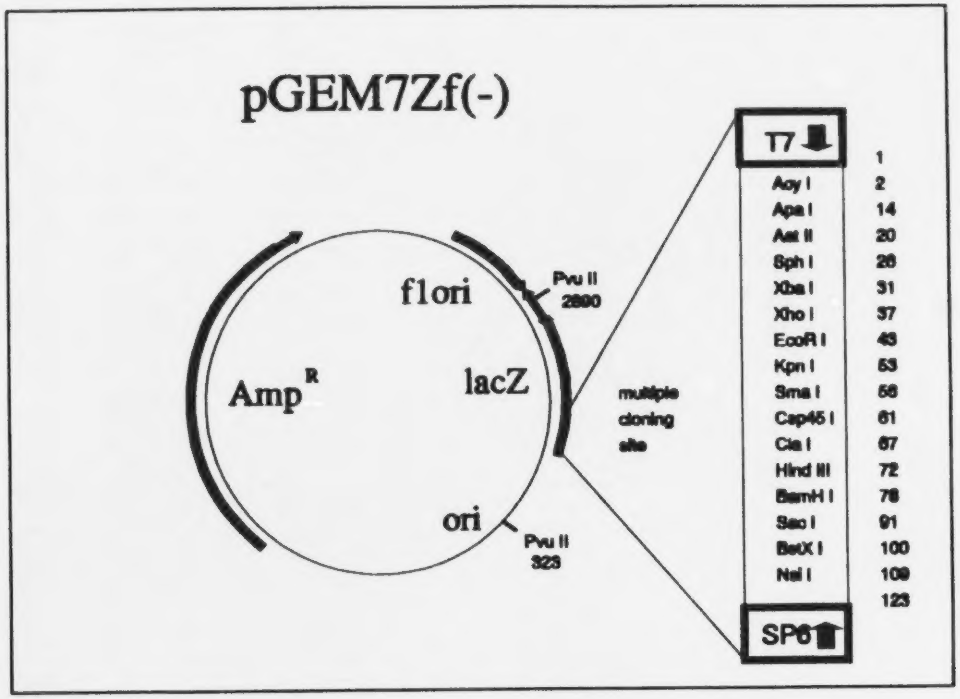

pGEM7Zf(-)
f1ori
Amp^R
lacZ
ori
Pvu II
2890
Pvu II
323
multiple
cloning
site
T7
Aoy I 2
Apa I 14
Aat II 20
Sph I 26
Xba I 31
Xho I 37
EcoR I 43
Kpn I 53
Sma I 56
Csp45 I 61
Cla I 67
Hind III 72
BamH I 78
Sac I 91
BstX I 100
Nsi I 109
1
123
SP6

FIGURE 32 The structure and relevant sequences of the pGEM-7Zf(-) vector (Promega). This vector is an M13-derived vector. The multiple cloning site is within the *lacZ* sequence and is flanked by the T7 and SP6 RNA polymerase promoters. The vector carries the *E. coli* and the *f*1 origins of replication. The reverse sequencing primer is a single-stranded 23-mer which anneals at positions 97-117. The 3'-OH group of this oligonucleotide can be utilized to primer DNA synthesis with any DNA polymerase. Numbers indicate predefined bp positions, with bp #1 corresponding to the T7 transcription start site. Several restriction enzyme sites, including all used in the present study, are indicated.

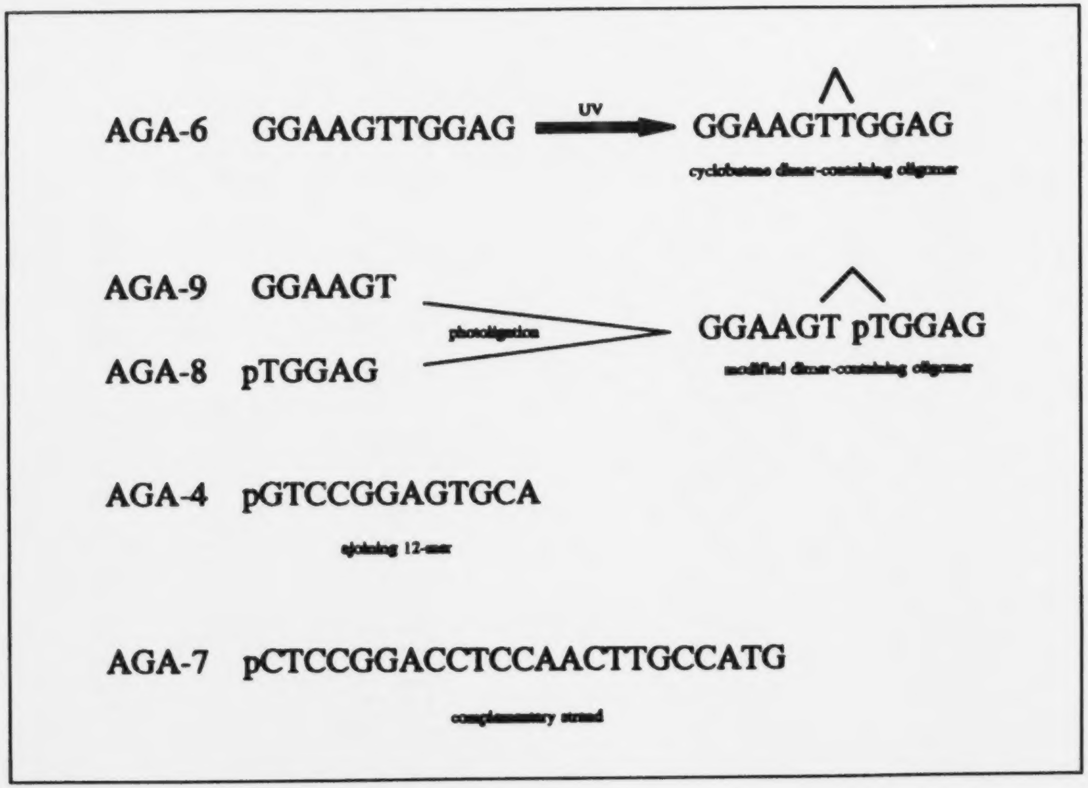

AGA-6
GGAAGTTGGAG
UV
GGAAGTTGGAG
cyclobutane dimer-containing oligomer
AGA-9
GGAAGT
AGA-8
pTGGAG
photoligation
GGAAGT pTGGAG
modified dimer-containing oligomer
AGA-4
pGTCCGGAGTGCA
adjoining 12-mer
AGA-7
pCTCCGGACCTCCAACTTGCCATG
complementary strand

FIGURE 33 Synthetic oligonucleotides used, in conjunction with pGEM-7Zf(-), to construct a double-stranded closed circular substrate with (i) no lesion, (ii) a cyclobutane dimer at a specific TT sequence, and (iii) a modified dimer at the same position.

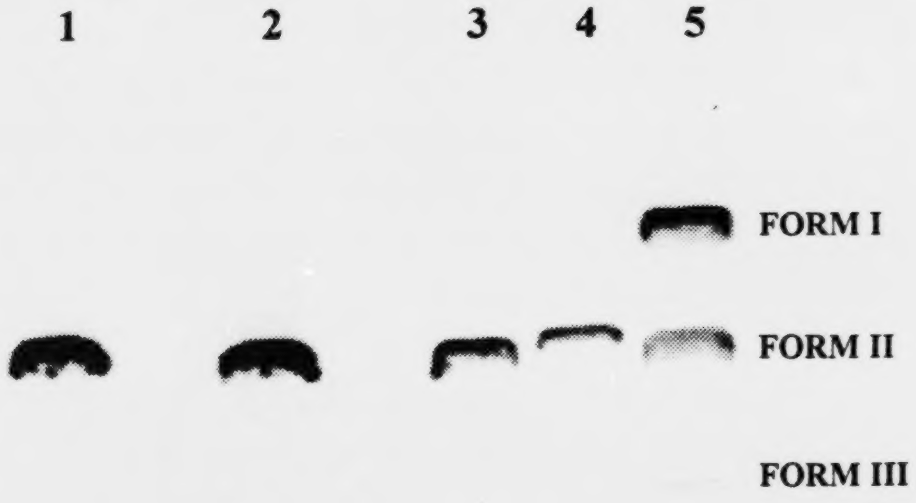


FIGURE 34 Agarose gel of pGEM-7Zf(-) before and after cutting with restriction enzymes. Lane 1: plasmid after double-digestion with *Nsi*I and *Sph*I; Lane 2: after cutting with *Nsi*I alone; Lane 3: after cutting with *Sph*I alone; Lane 4: as lane 1; Lane 5: uncut plasmid illustrating the positions of Form I (CCC), Form II (open-circle), and Form III (linear) plasmid.

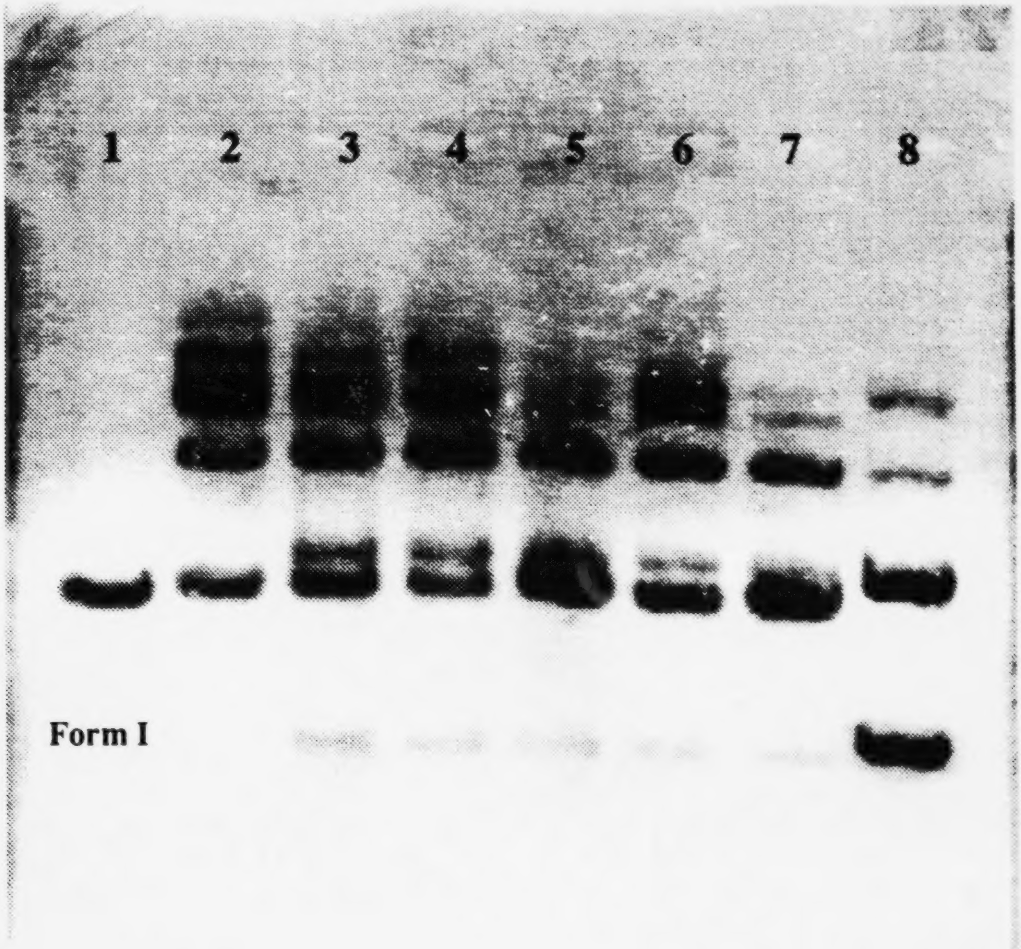


FIGURE 35 Agarose gel showing ligation products of reactions involving linearized pGEM-7Zf(-). Lane 1: *Nsi*I/*Sph*I-cut plasmid; Lane 2: ligation reaction with the 2917-bp fragment of *Nsi*I/*Sph*I-cut plasmid after separation of 2917- and 83-bp fragments by gel filtration; Lanes 3 and 4: re-ligated *Nsi*I-cut plasmid; Lanes 5 and 6: re-ligated *Sph*I-cut plasmid; Lane 7: re-ligated *Nsi*I/*Sph*I-cut plasmid without separation of 2917- and 83-bp fragments by gel filtration; Lane 8: uncut pGEM-7Zf(-). Re-ligation of plasmid results in regeneration of Form I DNA (indicated on diagram).

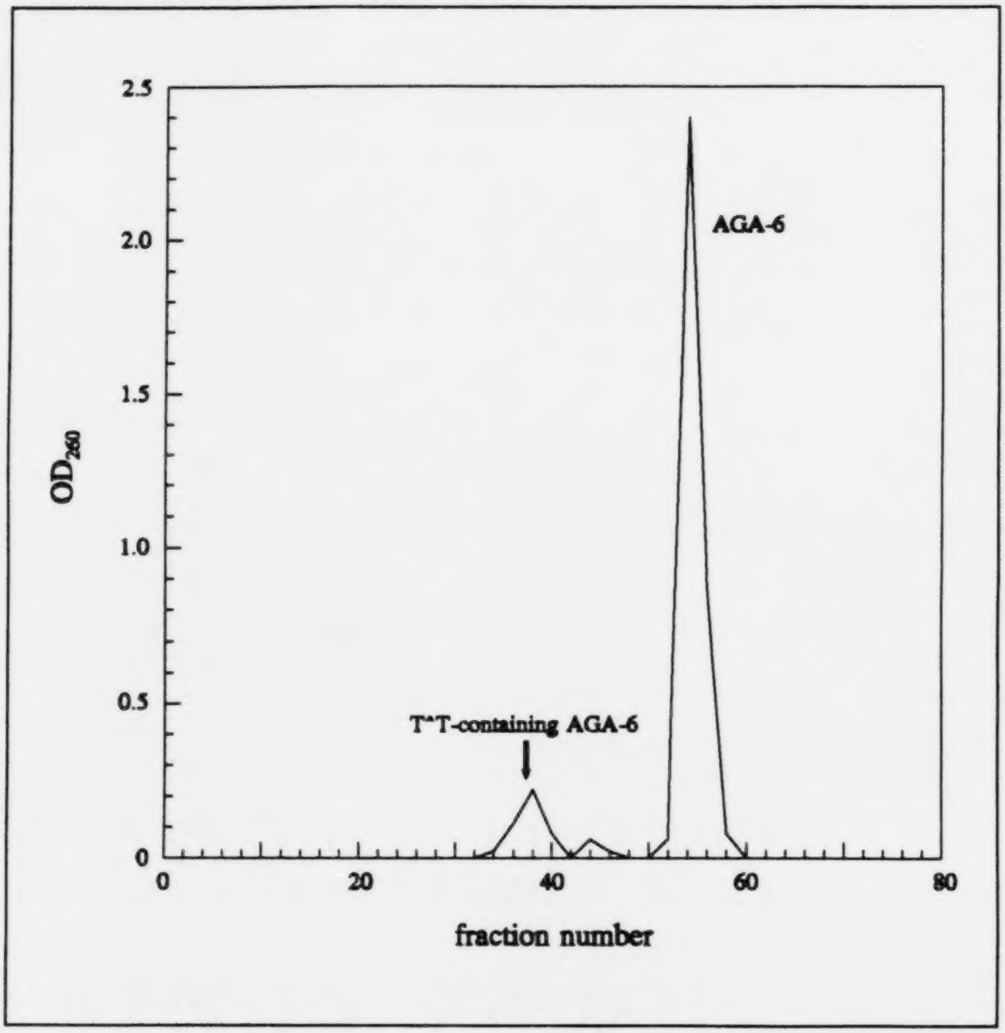


FIGURE 36 HPLC UV profile illustrating the separation of the T^T-containing AGA-6 from its parent molecule, by capped reverse-phase chromatography.

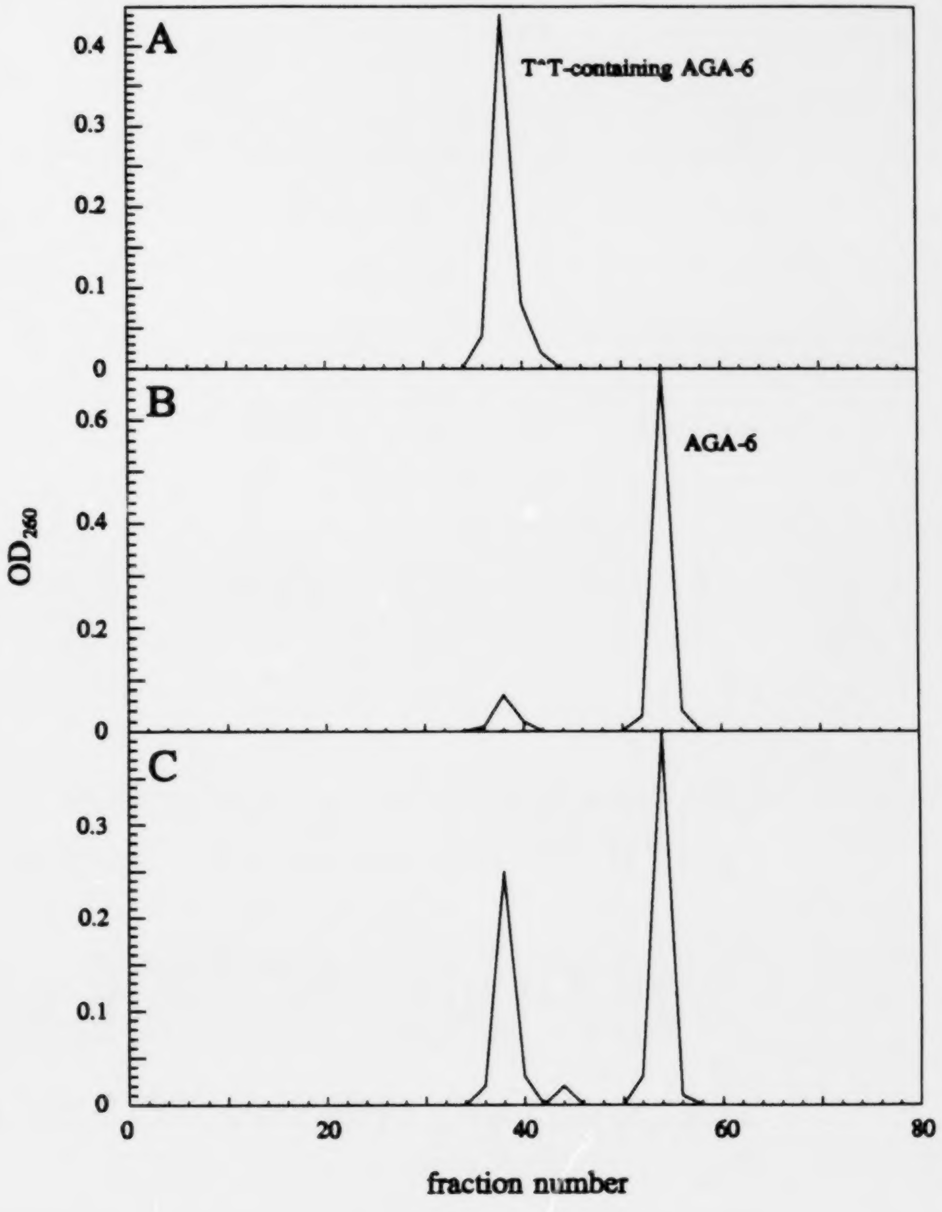


FIGURE 37 Reverse-phase HPLC UV profile displaying **A**: T^T-containing AGA-6; **B**: after treatment with photolyase; **C**: after photochemical reversal.

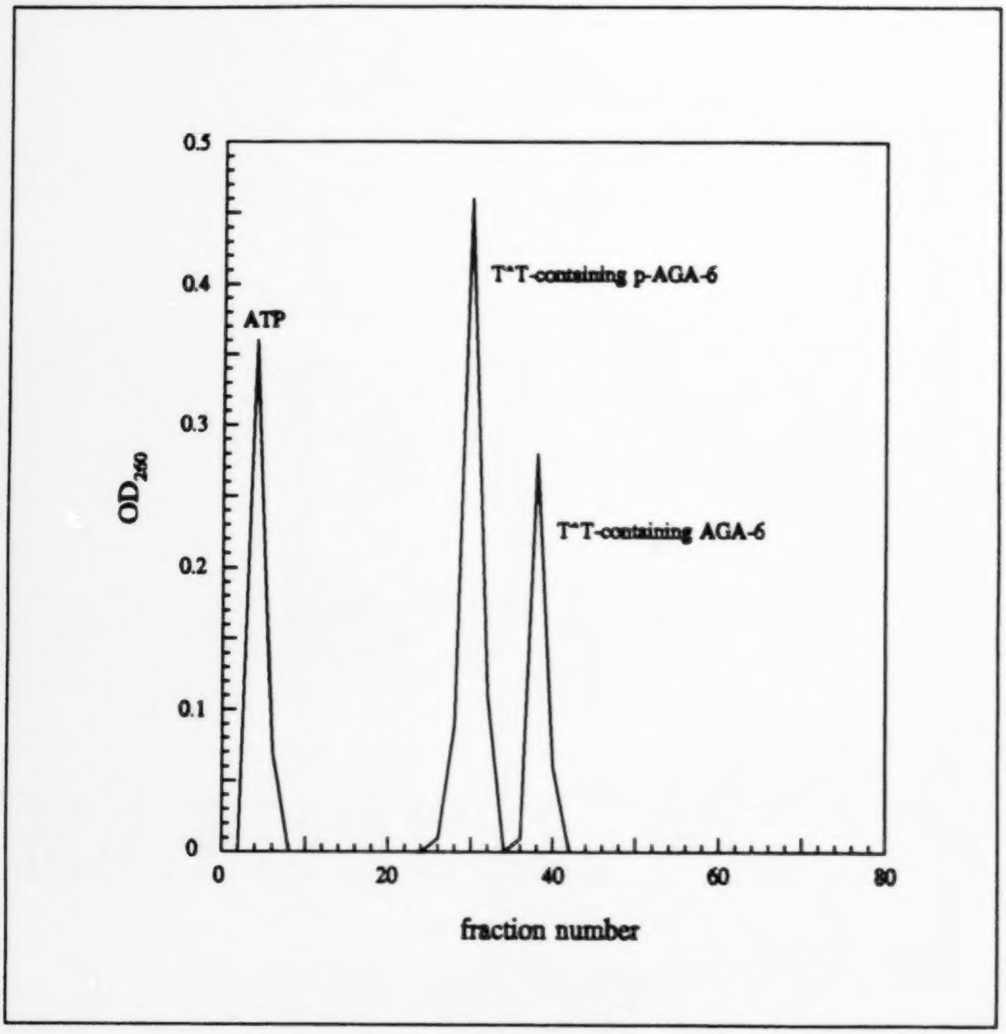


FIGURE 38 HPLC UV profile illustrating the separation of 5'-phosphorylated T^T-containing AGA-6 from the unphosphorylated molecule, by capped reverse-phase chromatography.

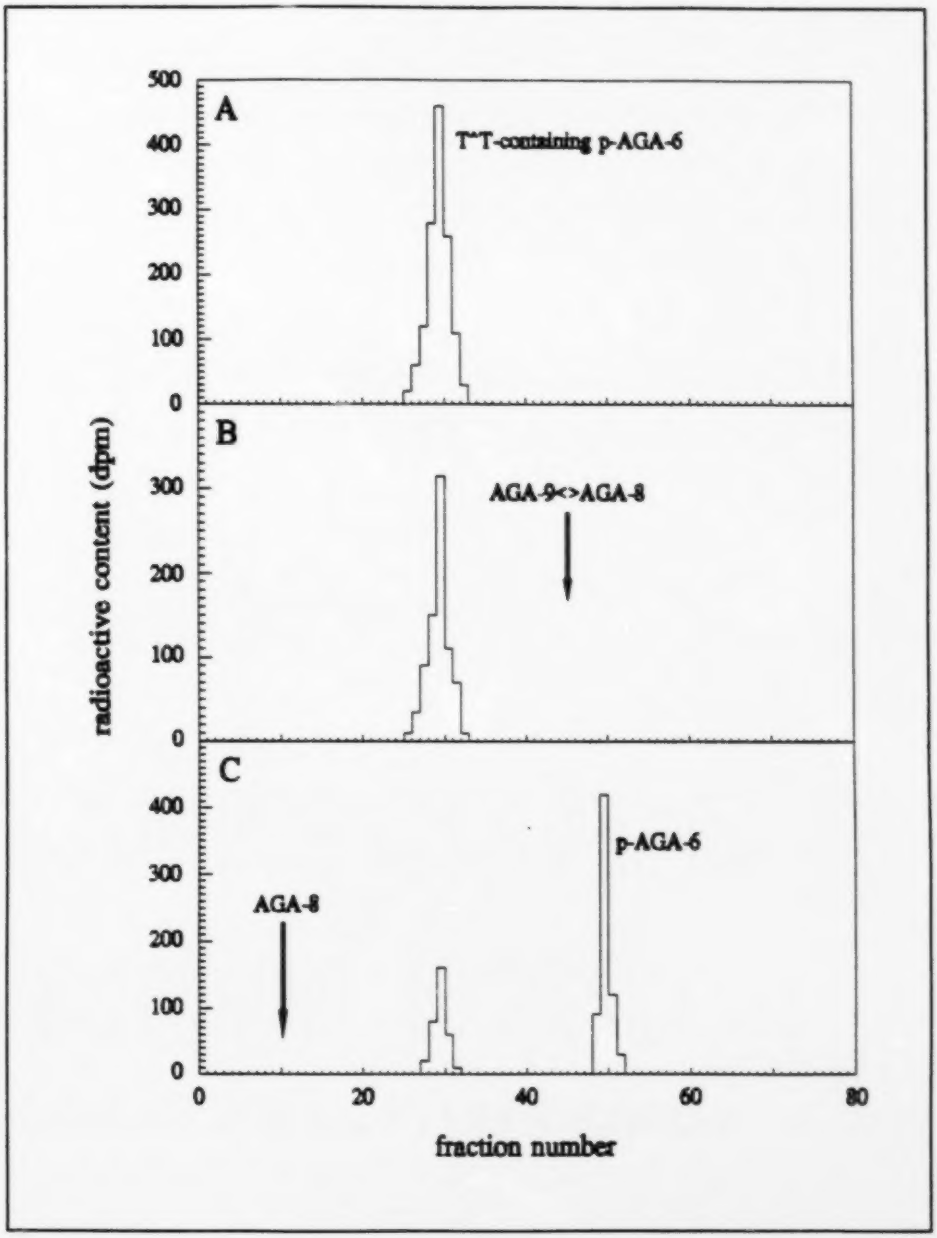


FIGURE 39 Reverse-phase HPLC chromatogram displaying the inactivity of IDP on T^T-containing AGA-6. **A:** [^{32}P] end-labelled T^T-containing AGA-6; **B:** after treatment with IDP; **C:** photochemical reversal following IDP treatment.

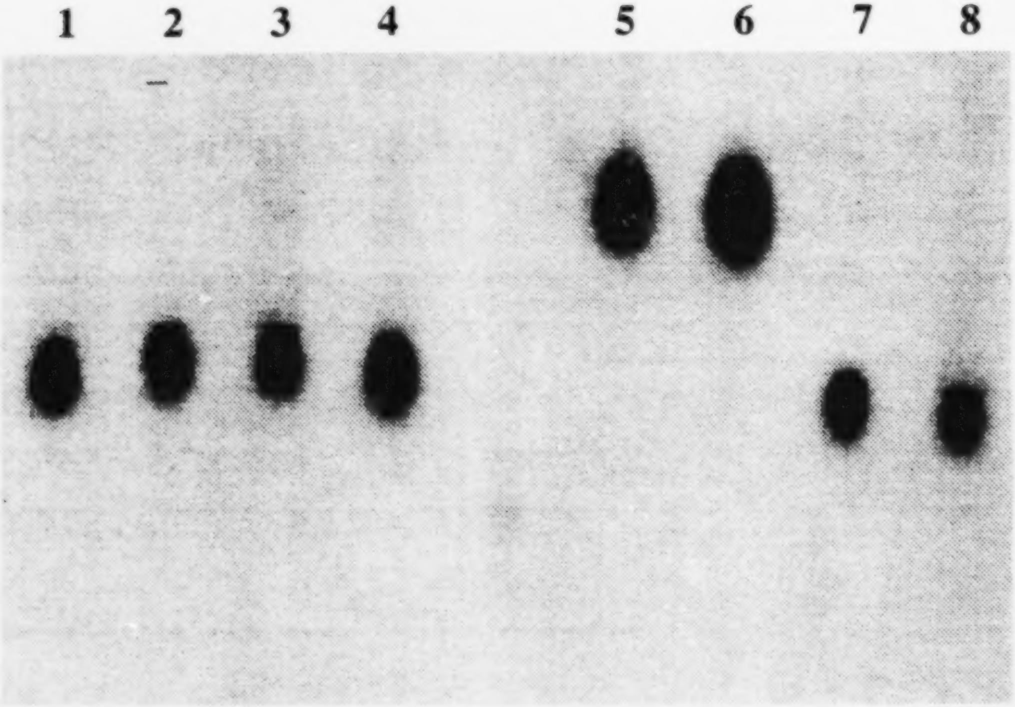


FIGURE 40 Polyacrylamide gel illustrating the inactivity of IDP on T^T-containing AGA-6. All oligonucleotides have been [^{32}P] end-labelled. Lane 1: T^T-containing AGA-6; Lane 2: T^T-containing AGA-6 incubated with human liver IDP; Lane 3: T^T-containing AGA-6 incubated with IDP, and subsequently treated with photolyase; Lane 4: T^T-containing AGA-6 incubated with IDP, and subsequently irradiated with a photochemical-reversing fluence (5 kJ/m^2) of 254-nm light; Lane 5: AGA-8; Lane 6: AGA-9; Lane 7: AGA-6; Lane 8: AGA-6 incubated with IDP.

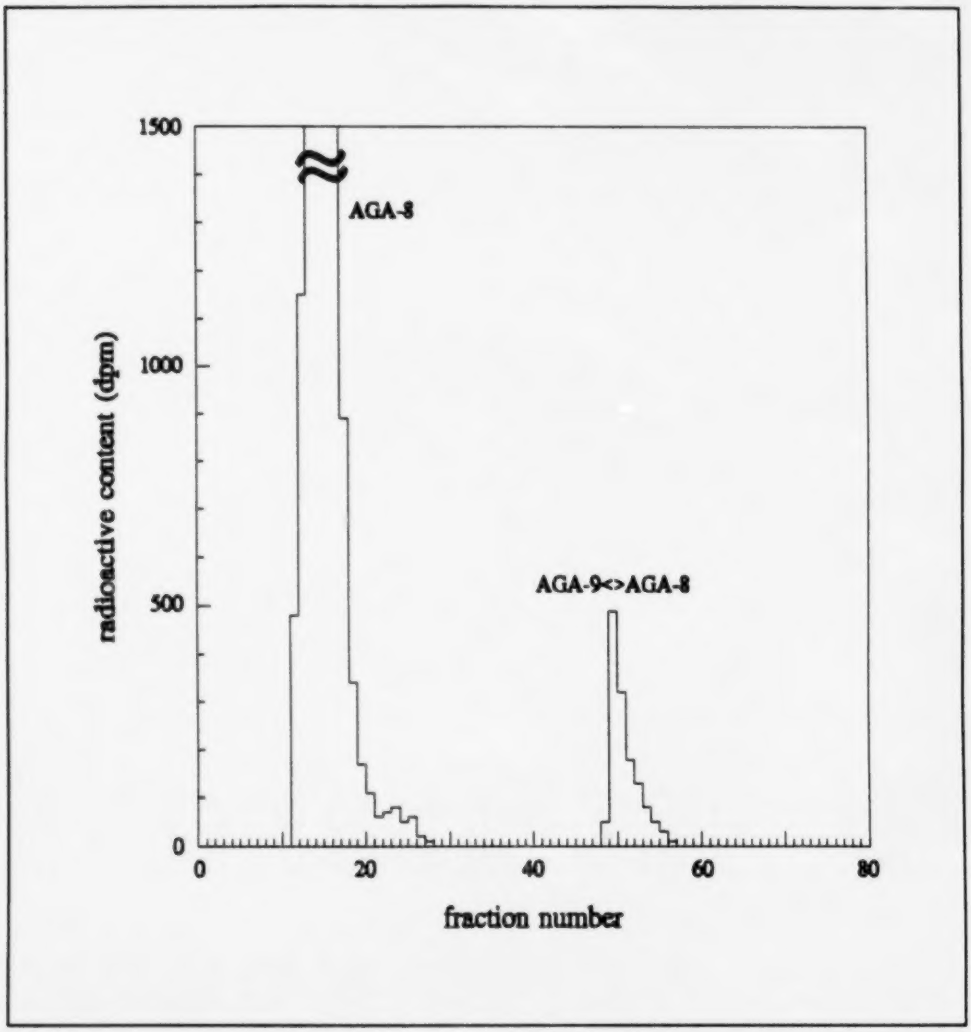


FIGURE 41 Reverse-phase HPLC chromatogram representing the isolation of a modified cyclobutane dimer-containing oligonucleotide generated by photoligating the two smaller oligomers, AGA-9 and AGA-8. Only AGA-8 is labelled in this experiment.

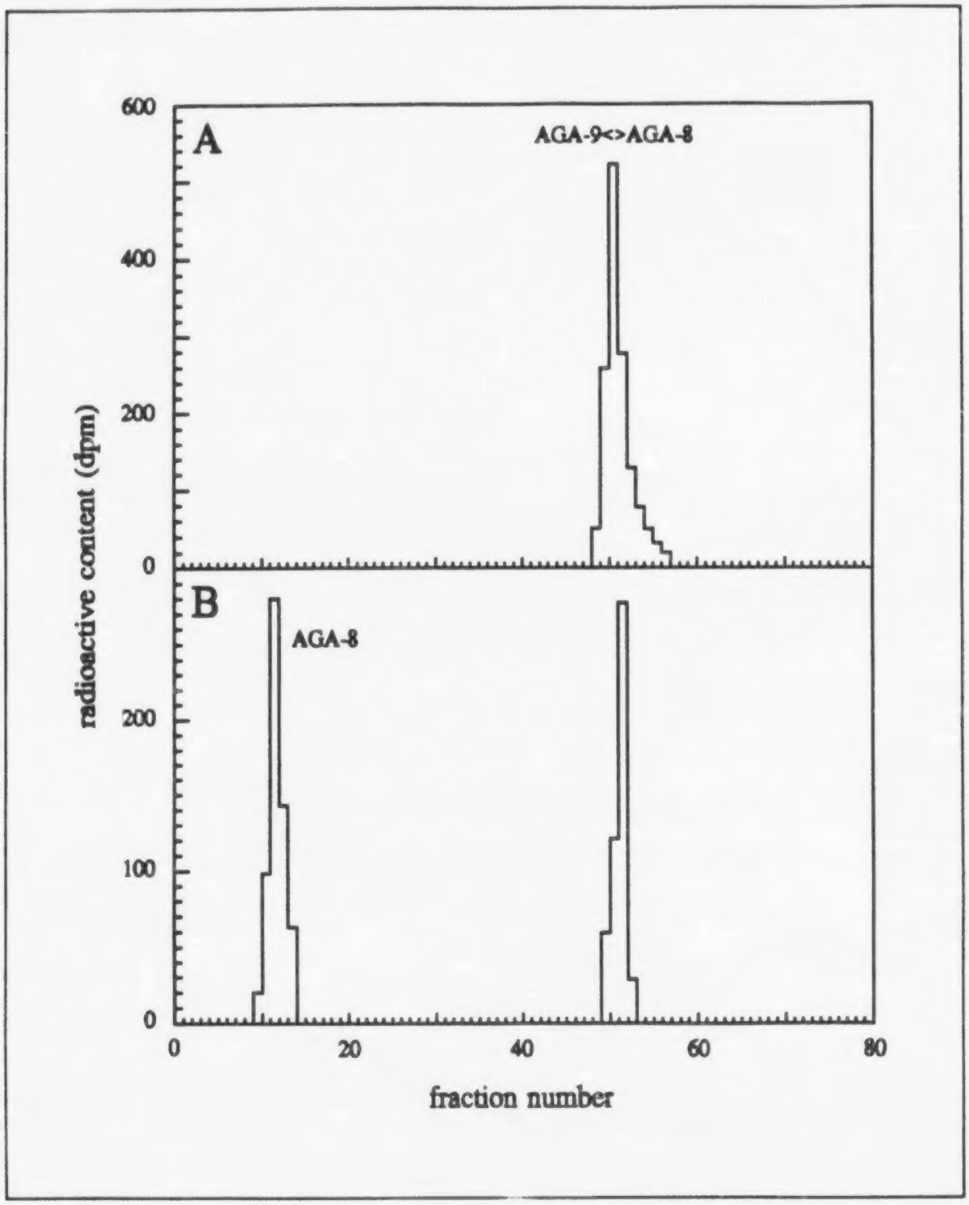


FIGURE 42 Reverse-phase HPLC chromatogram displaying **A**: photoligated oligonucleotides; **B**: after monomerization by photochemical reversal.

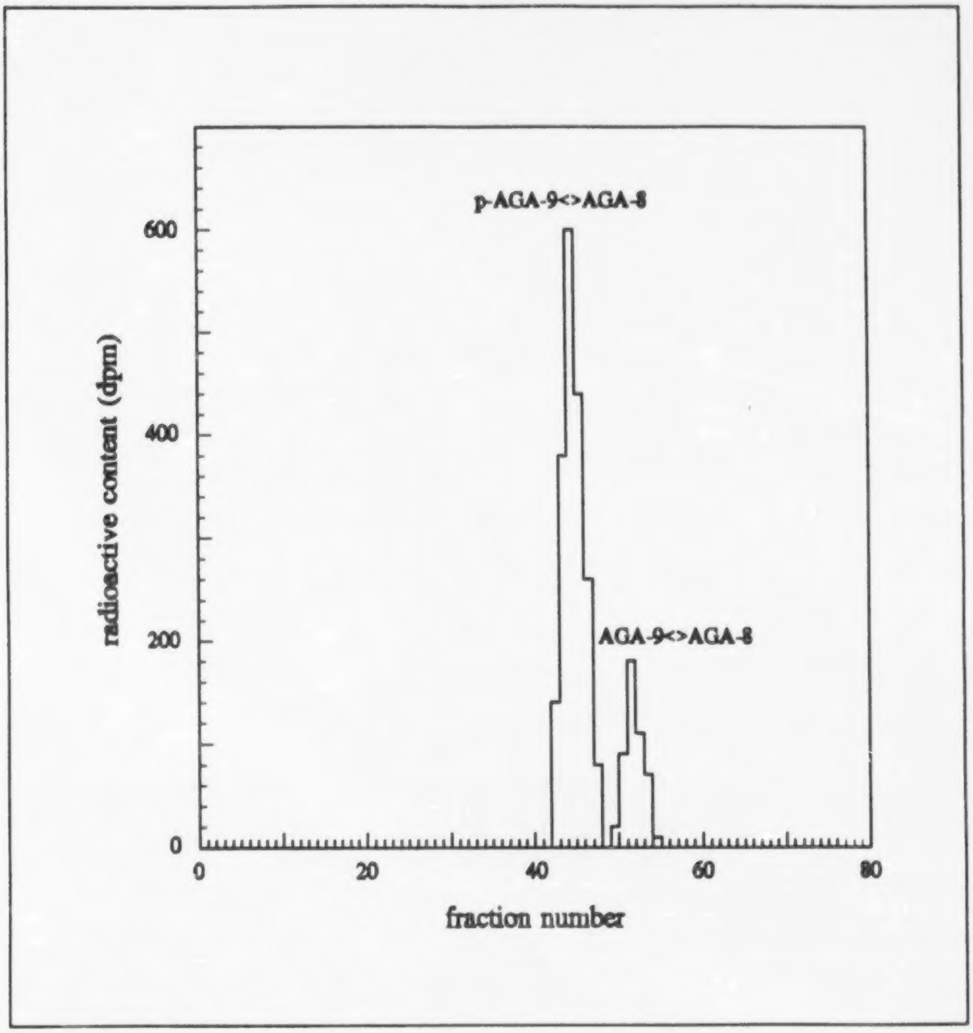


FIGURE 43 HPLC chromatogram illustrating the separation of the 5'-phosphorylated modified dimer-containing oligonucleotide from the unphosphorylated molecule, by capped reverse-phase chromatography.

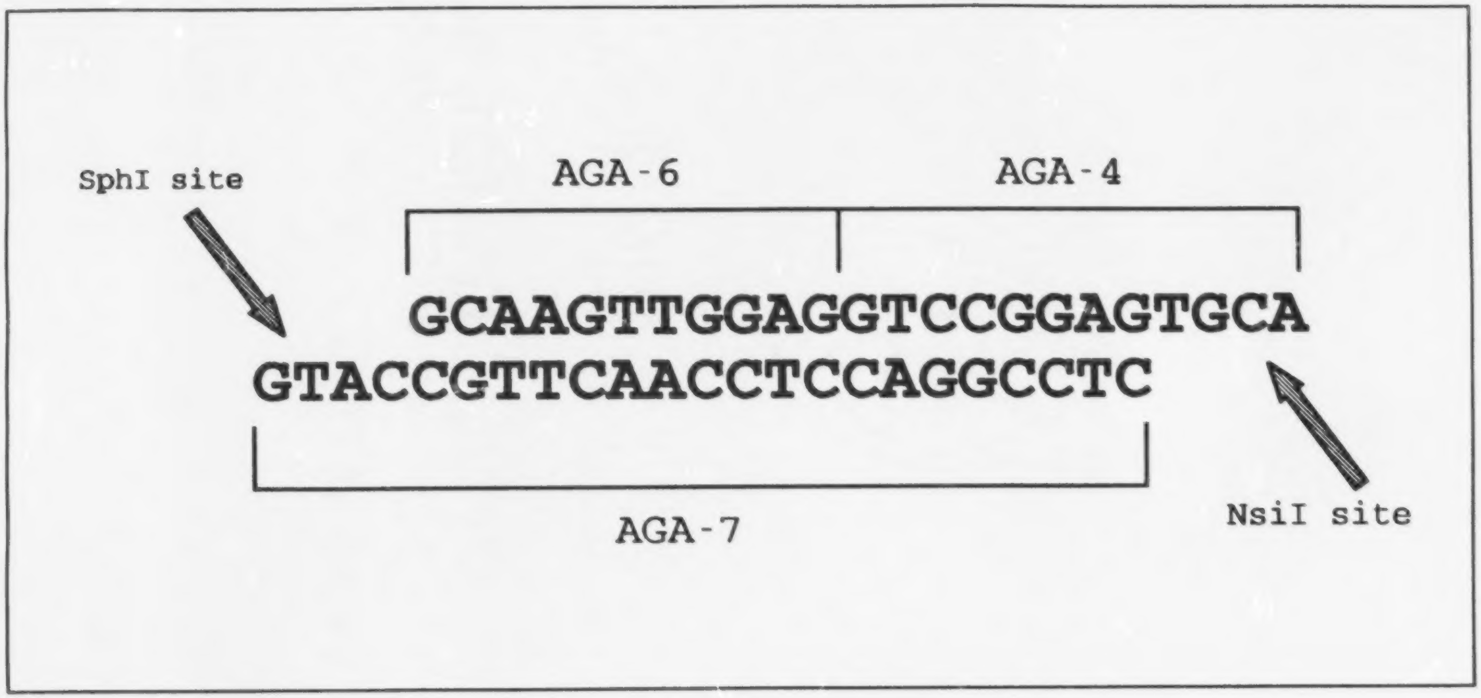

SphI site
AGA-6
AGA-4
GCAAGTTGGAGGTCCGGAGTGCA
GTACCGTTCAACCTCCAGGCCTC
AGA-7
NsiI site

FIGURE 44 Hybridized oligonucleotides, displaying the non-complementary ends.

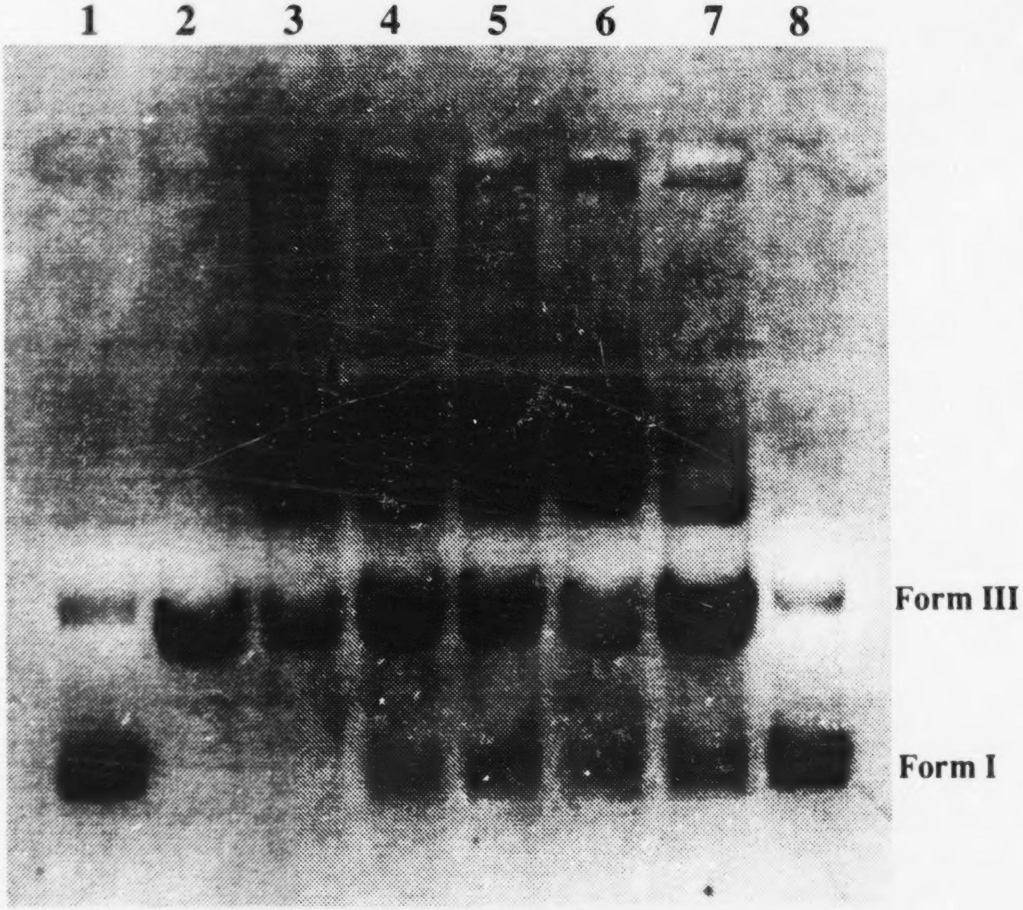


FIGURE 45 Agarose gel depicting the closed circular plasmid DNA generated by ligating oligonucleotides to the 2917-bp fragment of the pGEM-7Zf(-) vector. The gel was run in the presence of ethidium bromide. Lane 1: uncut pGEM-7Zf(-); Lane 2: 2917-bp fragment from *Nsi*I/*Sph*I-cut pGEM-7Zf(-); Lane 3: product of the ligation reaction involving only the 2917 bp *Nsi*I/*Sph*I fragment; Lane 4: products of the ligation reaction involving AGA-6, AGA-4, AGA-7, and the 2917-bp fragment of pGEM-7Zf(-); Lane 5: as lane 3, but using T^T-containing AGA-6 instead of AGA-6; Lane 6: as lane 3, but using AGA-9<>AGA-8 instead of AGA-6; Lane 7: products of the ligation reaction involving unpurified *Nsi*I/*Sph*I-cut pGEM-7Zf(-); Lane 8; as lane 1.

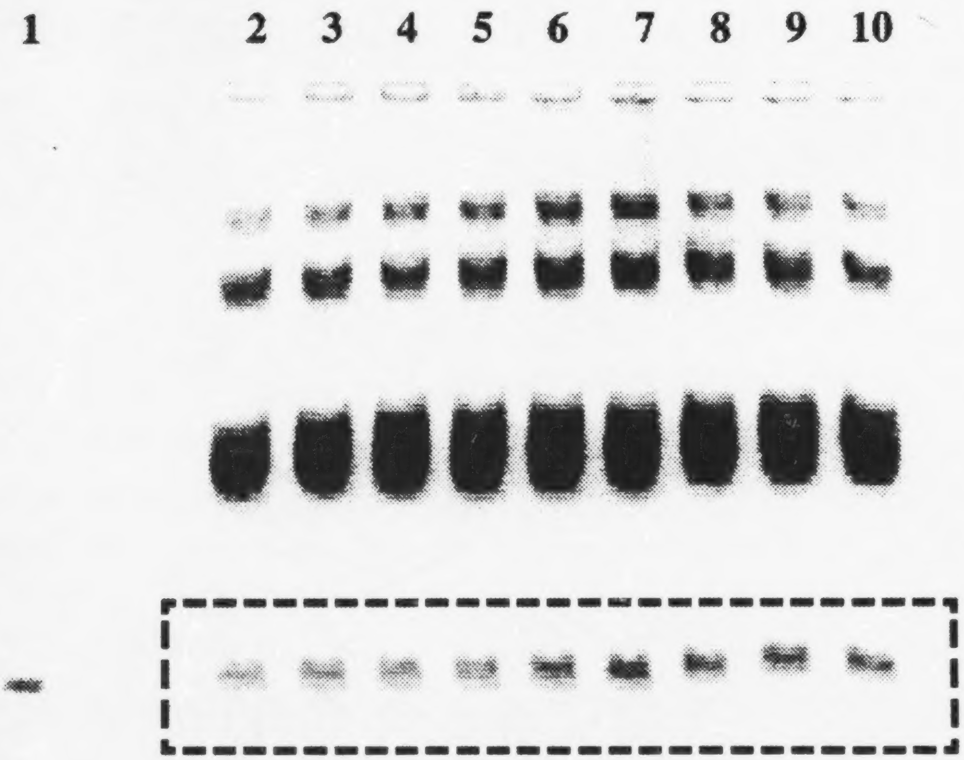


FIGURE 46 Low melt agarose gel, run in the presence of ethidium bromide, illustrating the products of a large scale ligation reaction involving T^T-containing AGA-6, AGA-4, AGA-7, and the 2917-bp *Nsi*I/*Sph*I fragment of pGEM-7Zf(-). Lane 1: uncut plasmid; Lanes 2 - 10: products of the ligation reaction. The bands indicated by the dashed box were cut from the gel, and the DNA was electroeluted.

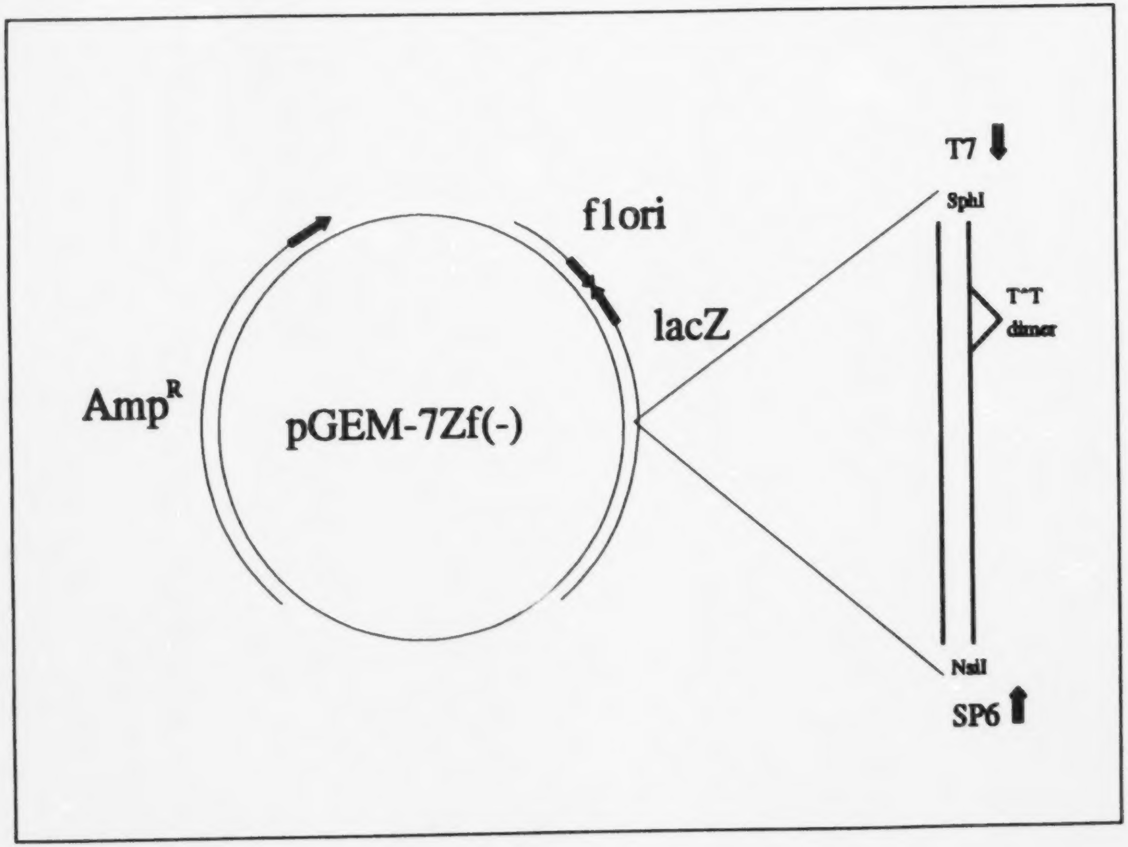

T7
SphI
T^T
dimer
NsiI
SP6
f1ori
lacZ
Amp^R
pGEM-7Zf(-)

FIGURE 47 Illustration of site-specific cyclobutane dimer-containing substrate generated by inserting a dimer-containing oligonucleotide into the multiple cloning site of the pGEM-7Zf(-) vector.

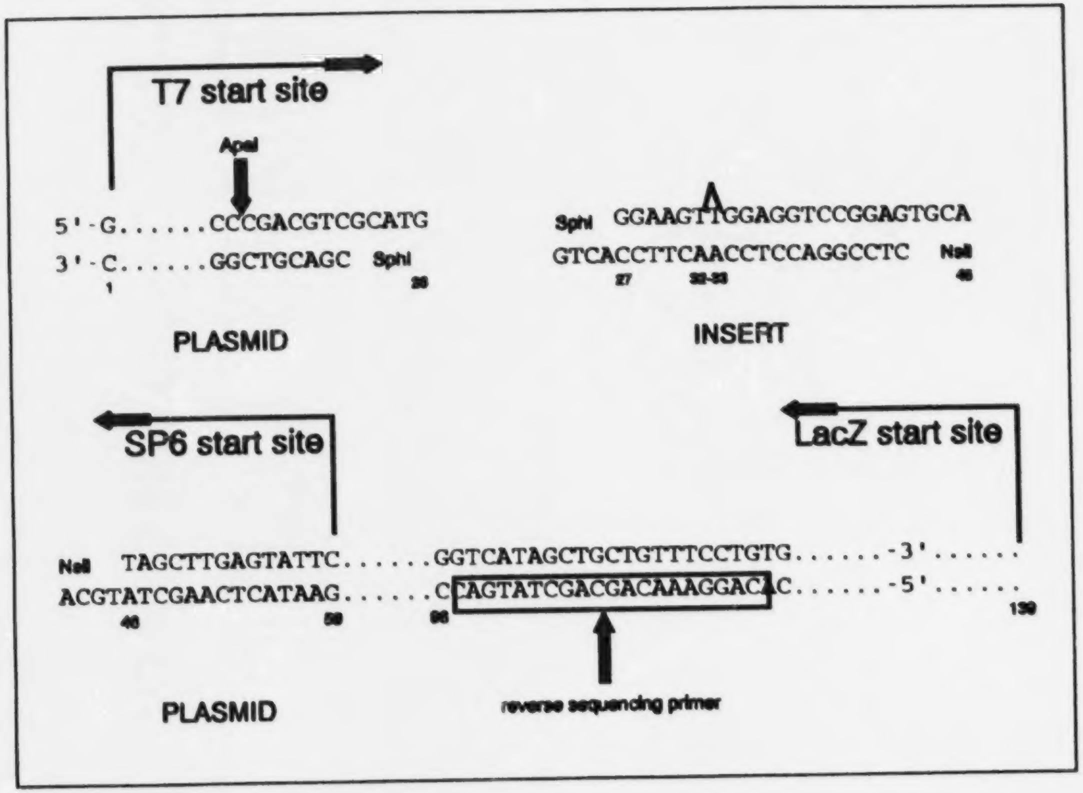

T7 start site
ApaI
5'-G......CCCGACGTCGCATG
3'-C......GGCTGCAGC SphI
1
26
PLASMID
SphI GGAAGTTGGAGGTCCGGAGTGCA
GTCACCTTCAACCTCCAGGCCTC NsiI
27
32-33
46
INSERT
SP6 start site
LacZ start site
NsiI TAGCTTGAGTATTC......GGTCATAGCTGCTGTTTCCTGTG......-3'......
ACGTATCGAACTCATAAG......CCAGTATCGACGACAAAGGACAC......-5'......
46
59
96
139
PLASMID
reverse sequencing primer

FIGURE 48 Detailed representation of sequences relevant to *in vitro* replication and transcription in constructed substrate. Although this diagram depicts a cyclobutane dimer at position 32-33, substrates containing either no lesion, or a modified cyclobutane dimers at this position, were also constructed. The T7 start site is designated as position 1.

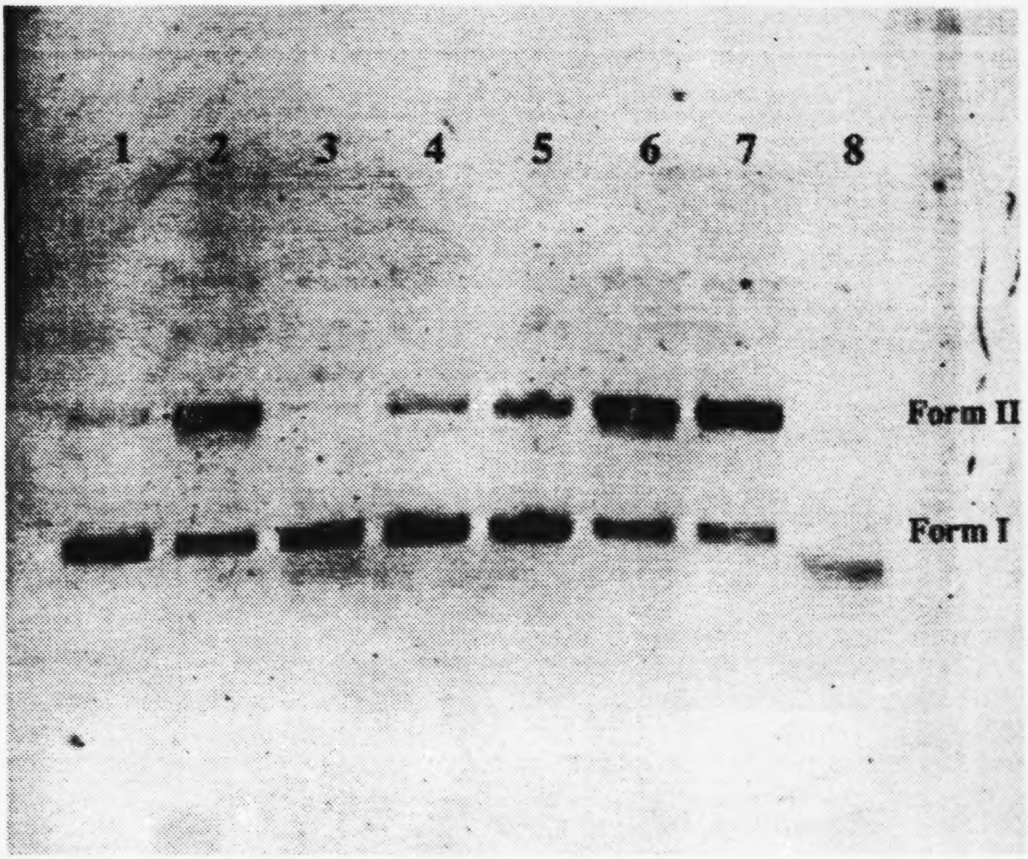


FIGURE 49 Agarose gel displaying the activity of 3 independent DenV preparations on irradiated and unirradiated plasmid DNA. Lane 1: unirradiated pGEM-7Zf(-) plasmid; Lane 2: unirradiated plasmid treated with DenV prep#1; Lane 3: unirradiated plasmid treated with DenV prep#2; Lane 4: unirradiated plasmid treated with DenV prep#3; Lane 5: UV-irradiated (2 kJ/m^2 of 254-nm light) plasmid; Lane 6: irradiated plasmid treated with DenV prep#1; Lane 7: irradiated plasmid treated with DenV prep#2; Lane 8: irradiated plasmid treated with DenV prep#3.

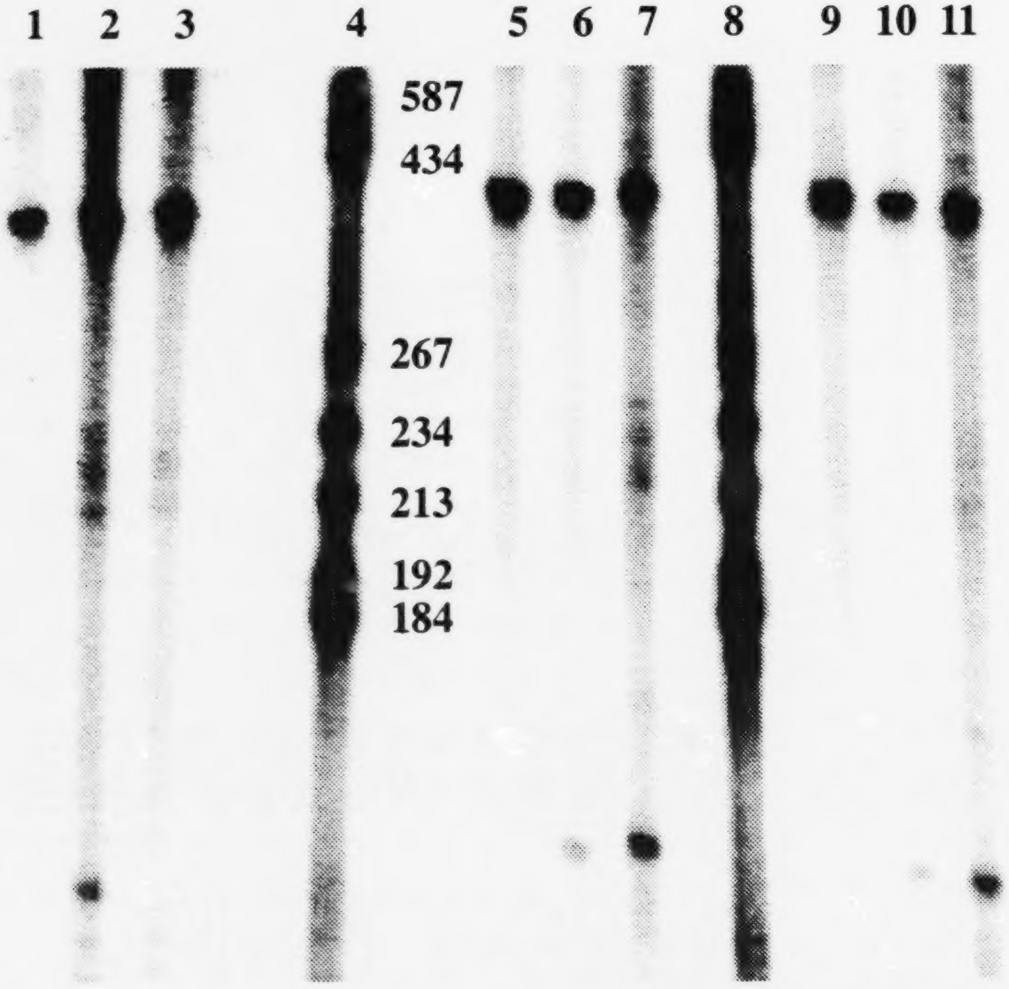


FIGURE 50 Polyacrylamide gel displaying the activity of T4 UV endonuclease (DenV) on the pGEM-derived constructs. All substrates have been linearized with *Pvu*II and [^{32}P] end-labelled. The amount of DenV used in these experiments represents a 2-fold excess over that required to incise every cyclobutane dimer present in ID-pGEM. Lane 1: m-pGEM; Lane 2: MD-pGEM; Lane 3: ID-pGEM; Lane 4: marker DNA; Lane 5: m-pGEM treated with DenV; Lane 6: MD-pGEM treated with DenV; Lane 7: ID-pGEM treated with DenV; Lane 8: marker DNA; Lane 9: m-pGEM hydrolysed with human liver IDP prior to DenV treatment; Lane 10: ID-pGEM hydrolysed with IDP prior to DenV treatment; Lane 11: ID-pGEM treated with DenV.

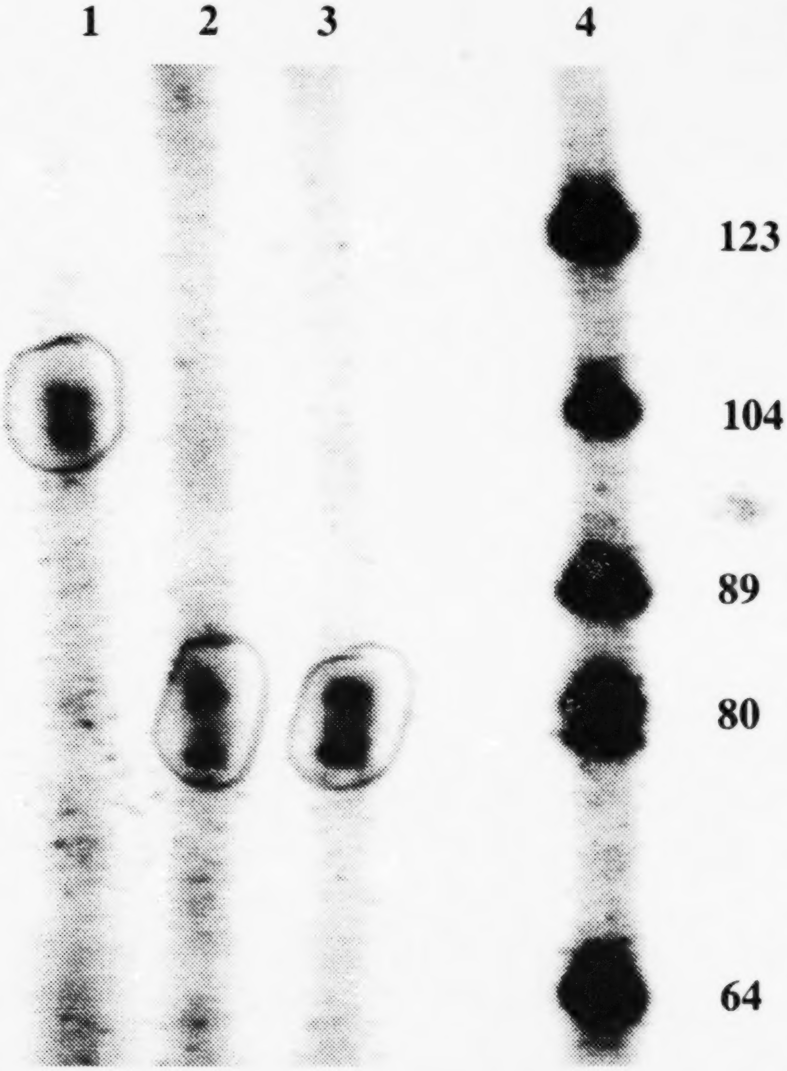


FIGURE 51 Polyacrylamide denaturing gel illustrating *in vitro* replication of the pGEM-derived constructs by *E. coli* DNA polymerase I. Replication begins from the reverse sequencing primer which anneals at nucleotides 96 - 113 (see Figure 32). All constructs have been previously linearized with *Apa*I. Lane 1: m-pGEM; Lane 2: ID-pGEM; Lane 3: MD-pGEM; Lane 4: marker DNA. Sizes of markers are indicated.

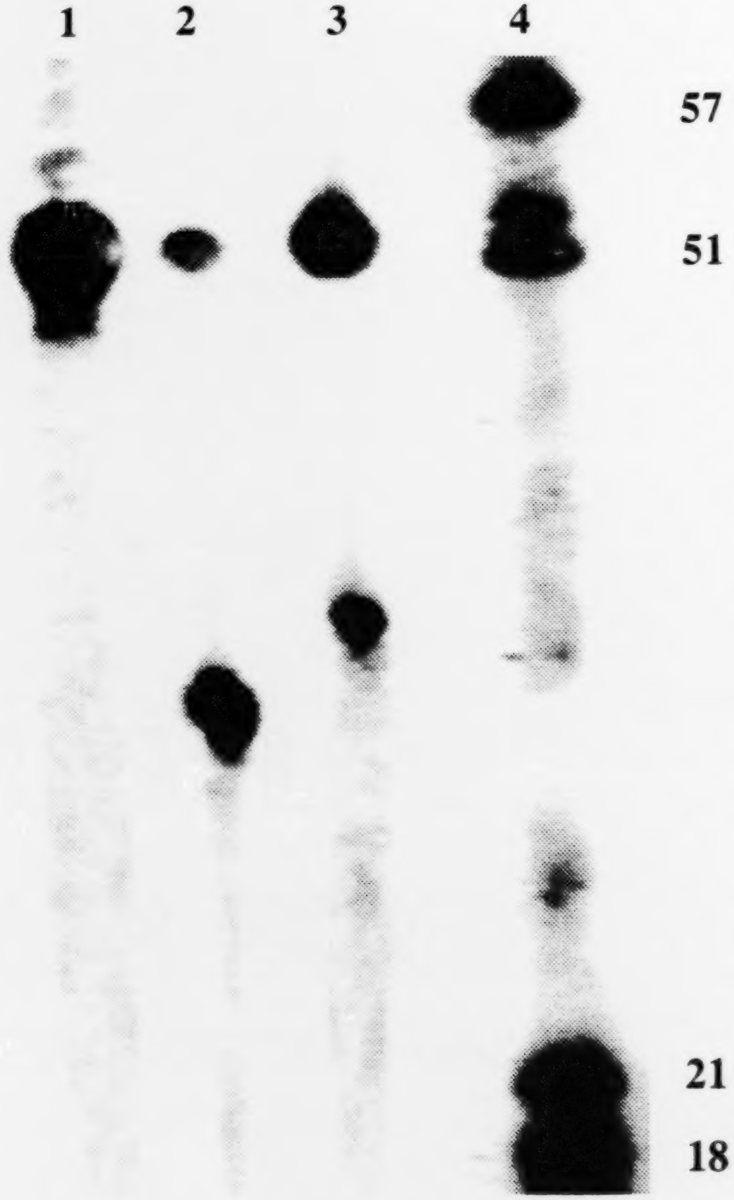


FIGURE 52 Polyacrylamide denaturing gel illustrating *in vitro* transcription of the pGEM-derived constructswith SP6 RNA polymerase. All substrates have been linearized with *Apa*I. Lane 1: m-pGEM; Lane 2: ID-pGEM; Lane 3: MD-pGEM; Lane 4: marker DNA. Sizes of markers are indicated.

CHAPTER NINE - SUMMARY, GENERAL CONCLUSIONS and FUTURE DIRECTIONS

The novel three stage enzymatic hydrolysis / HPLC protocol, which has been introduced in this dissertation, was devised as a technique to quantitate the induction and repair of UV-induced DNA damage and is considered to be a valuable alternative to preexisting procedures. This new approach, which entails: (i) isolation of [^{3}H] thymidine-labelled excision fragments or genomic DNA from post-UV (40 J/m^2, 254-nm light) incubated human cells; (ii) formic acid hydrolysis; (iii) digestion with snake venom phosphodiesterase, nuclease P1, and calf alkaline phosphatase; and (iv) hot alkali treatment of enzymatically digested oligonucleotides, has allowed the direct measurement of UV lesions contained within both genomic DNA and the oligonucleotides excised by a living cell during nucleotide excision repair. Furthermore, this methodology has permitted independent and accurate quantitation of several classes of UV photoproducts in a single assay (including unmodified cyclobutane dimers, cyclobutane dimers with a severed interpyrimidine phosphodiester bond, (6-4) photoproducts, and the recently discovered TA* photoproduct) thus circumventing a major shortcoming of conventional techniques used to assess UV-induced DNA damage.

Established DNA repair assays either detect only one specific type of UV-induced damage or, alternatively, measure general repair without discrimination of lesion type. For example, UV endonuclease site removal (Paterson *et al.*, 1981), formic acid hydrolysis (Love and Friedberg, 1983), and radioimmunoassays utilizing antibodies directed towards a cyclobutyl ring (Mitchell *et al.*, 1982; Mitchell and Nairn, 1987; Mitchell *et al.*, 1990c), represent measurement techniques specific for cyclobutane dimers. Neither T4 nor *M. luteus* UV endonuclease has affinity for (6-4) photoproducts (Gordon and Haseltine, 1980), and this latter class of UV adducts is not stable under the conditions associated with formic acid hydrolysis (Wang and Varghese, 1967). These three procedures also give no information as to the status of the intradimer phosphodiester bond, and in fact, the discriminatory power of a radioimmunoassay may be compromised by cleavage of this bond (Mitchell *et al.*, 1982; Mitchell and Nairn, 1987).

The use of antibodies directed towards (6-4) photoproducts (Mitchell *et al.*, 1985) and the enumeration of alkali labile sites (Mitchell, 1988b) have been successfully employed to measure the induction and repair of (6-4) photoproducts. However, these procedures give no information regarding cyclobutane dimers. In addition, it appears as though TA* may also be alkali labile (see Chapter 2, page 31 for discussion on data presented in Myles *et al.*, 1987), and therefore the latter protocol may neither be specific for (6-4) photoproducts nor capable of distinguishing between these two classes of UV lesions.

A different type of assay used to ascertain the repair capacity of a UV-irradiated cell measures repair synthesis. Several distinct techniques have been developed for this purpose including, (i) measurement of unscheduled DNA synthesis by autoradiography (Cleaver and Thomas, 1981), (ii) incorporation of bromouracil into repair patches and assessment of their incidence by equilibrium centrifugation (Smith *et al.*, 1981), and (iii) incorporation of bromouracil followed by photolysis to indicate the frequency of repair patches (Setlow and Regan, 1981). These are general means of assessing the total repair capacity of a cell. As well, they are comparative assays, where an experimental cell strain is compared to a 'normal' strain of the same cell type under the same conditions. None of these procedures provides information with regards to either the amount of UV damage induced or the class(es) of lesions being repaired.

Given the ability of the enzymatic hydrolysis / HPLC protocol to accurately quantitate all major types of UV-induced DNA damage in both genomic DNA and excision fragments, it is felt that this is the methodology of choice. Its one apparent shortcoming, the ability to detect only thymine-containing photoproducts, can be easily overcome by radioactive labelling of genomic DNA with either a different [^{3}H]-labelled base or, alternatively, by double-labelling with both a [^{3}H]-labelled base and a [^{14}C]-labelled base. As a case in point, the induction and repair of both C^C cyclobutane dimers and C[p]C (6-4) photoproducts have been approximated in this current work. Although these estimations have been based on the relative induction of cytosine-cytosine lesions with respect to thymine-containing UV adducts as reported by others, these values need not accurately reflect the rate of excision. As an example, as has been presented in this dissertation, although T^T cyclobutane dimers were induced at the higher rate, T^C dimer repair occurred more rapidly. Consequently, double-labelling with [^{3}H] thymine and [^{14}C] cytosine would offer both a firsthand method of quantitating cytosine-containing photoproducts, and a direct comparison to thymidine-containing adducts.

Using the enzymatic hydrolysis / HPLC methodology, the repair capacity of normal human fibroblasts, fibroblast strains representing the eight complementation groups of xeroderma pigmentosum (XP), the three classes of trichothiodystrophy (TTD), as well as those established from two patients with pronounced solar sensitivity of unknown origin, normal and UV-sensitive Chinese hamster ovary cell lines, and wild-type and mutant *E. coli* strains, have been assessed. For normal human fibroblasts and normal Chinese hamster ovary cells, results obtained from this new protocol are in keeping with published data for cyclobutane dimer and (6-4) photoproduct induction and repair [Paterson *et al.*, 1973; Meyn *et al.*, 1974; Paterson *et al.*, 1981; Yagi, 1982; Vijg, *et al.*, 1984; Mitchell *et al.*, 1985; Mitchell and Nairn, 1987; see Chapter 2, page 30 for discussion on the various rates of (6-4) photoproduct induction which have been reported]. Furthermore, the disappearance of lesions from genomic DNA can be accounted for by their accumulation in excision fragments. These concordances offer critical support for the accuracy and competency of this new method.

In agreement with other reports (Mitchell *et al.*, 1985; Mitchell and Nairn, 1989), (6-4) photoproduct excision was essentially complete by 6 hr in both normal human and rodent cells treated with 40 J/m^2. In both instances, removal of the more prevalent cyclobutane dimers was protracted by comparison, but this was much more extreme in rodent cells which excised only ~4% of these adducts in 24 hr following UV irradiation, compared to ~25% in human fibroblasts. Because of this marked difference in the repair rate of these two classes of UV adducts, at 2 hr post UV, the time at which UDS in typically assessed, (6-4) photoproduct repair (as well as TA* photoproduct removal; see below) would account for ~80% of unscheduled DNA synthesis in normal human fibroblasts and essentially 100% in CHO cells. The competency of rodent cells to remove (6-4) photoproducts, while being nearly inept at cyclobutane dimer removal, has been offered as an explanation for their high UV survival (Mitchell *et al.*, 1985; Mitchell *et al.*, 1988). This idea is in keeping with the premise that this class of UV photoproducts represents the major cytotoxic UV-induced DNA damage (Mitchell *et al.*, 1985; Mitchell, 1988a; Cleaver *et al.*, 1988; Cleaver, 1989).

At the time of the enzymatic hydrolysis / HPLC assay development, little work had been done on assessing the repair of (6-4) photoproducts in xeroderma pigmentosum cells. Although all eight complementation groups of XP have been examined, the primary aim of this study was to reveal information which could explain the apparent discrepancies in repair data for XP groups D and F. XP-D cells appear to be much more repair proficient when assessed by UDS (20-55% of normal; Kraemer, 1983) than by UV endonuclease site removal (~0% of normal; Zelle and Lohman, 1979). XP-F cells show the opposite disparity (Kraemer, 1983; Zelle and Lohman, 1979). This investigation not only solved this dilemma, but two general and important conclusions could be drawn from it. Firstly, each XP complementation group exhibited a unique proficiency / deficiency in the repair of cyclobutyl dimers and/or (6-4) photoproducts (with the exception of groups A and G which both lacked repair of both classes of UV lesions). The repair of one class of these UV adducts appeared to be completely independent of the repair of the second. This not only implies that mutations at different loci underlie the various genetic forms of XP, but in addition, suggests that cyclobutane dimers and (6-4) photoproducts have separate mechanisms in their respective repair pathways, most likely at the recognition stage. These pathways must again converge, however, as a single mutation often affects the repair of both types of UV damage. Secondly, the difference in repair of these two major UV photoproducts directly accounts for the level of UDS seen in all cases. Since (6-4) photoproduct repair was estimated to account for ~80% of UDS at 2 hr in normal cells, this, along with the proficiency of repair of each lesion type, could be used to calculate expected UDS. For XP group D cells, their reduced (6-4) photoproduct repair (40% of normal), along with their total inability to remove cyclobutane dimers, corresponds to a calculated UDS of 32%, in excellent agreement with published results (20-55%; Kraemer, 1983). XP group F cells, on the other hand, were normal for cyclobutane dimer removal, but completely inept at repairing (6-4) photoproducts. This correlates to a calculated UDS of ~20%, also in excellent agreement with the findings of others (Kraemer, 1983). For the remaining six XP complementation groups, although there is no discrepancy in their estimated repair proficiency whether ascertained by UDS or UV endonuclease site removal, UDS calculated in this manner still correlates extremely well with the observed values.

This investigation was then extended to include other human UV-sensitive cell strains, most notably those originating from TTD patients. Fibroblast strains derived form these patients may or may not display photosensitivity. The UV-sensitive phenotype has been attributed to a defect in the XP-D gene on the basis of the inability of these cells to complement the repair defect in TTD cells from classes 2 and 3 (Stefanini *et al.*, 1986). Trichothiodystrophy has been divided into three classes. Cell strains representing the first class do not exhibit a UV-sensitive phenotype and, as expected, are without any detectable repair defects. The repair-deficient strains have been separated into classes 2 and 3 on the basis of differences in their repair properties. One fibroblast strain representing each of these three classes of TTD has been assessed, using the new enzymatic hydrolysis / HPLC technique, to allow direct comparison of the repair deficiency in TTD groups 2 and 3 to that in XP-D cells. From this study, it was obvious that the repair deficiency in both class 2 and 3 TTD cell strains was markedly different from that of XP-D cells. XP group D cells displayed about 40% of normal (6-4) photoproduct repair, with kinetics that suggest domain limited repair, and were completely unable to excise cyclobutane dimers. However, TTD group 2 cells, following 40 J/m^2 of 254-nm light, displayed about one-third of normal repair capacity for the latter class of lesions and removed (6-4) photoproducts completely, albeit with reduced kinetics. TTD group 3 cells were much more XP-D like in that they were unable to repair cyclobutane dimers and had a reduced capacity to remove (6-4) photoproducts. However, (6-4) photoproducts were repaired in these cells with kinetics unlike those displayed by XP-D cells. This challenges the relationship of the XP-D and TTD genes. It has been proposed that the UV-sensitive phenotype of some classes of TTD results from deletions that extend from the TTD gene into the neighboring XP-D locus. However, if this were the case, one would expect to find XP-D cell strains with repair characteristics similar to those of the repair-deficient TTD cell strains. Although only one XP group D fibroblast strain was examined in this current work, there has not been a report of XP-D cells capable of a significant amount of cyclobutane dimer repair, as are the non-complementing TTD group 2 cells. An alternative hypothesis which warrants consideration is that the mutant gene products, when present in combination (as is the case in a heterokaryon derived from cell-cell fusion of a TTD cell and an XP-D cell), interact in an antagonistic manner with the normal gene products thereby nullifying normal repair capabilities.

Enzymatic hydrolysis of genomic DNA or excision fragments isolated from post-UV incubated cells has also offered assessment of the induction and repair of TA^* photoproducts. Although this minor class of UV lesions was first described in 1983 (Bose *et al.*, 1983), there has been no attempt made to assess its repair in any cell type. This is likely due, in large, to its presumed insignificant occurrence in DNA (~1% the frequency of cyclobutane dimers) (Bose *et al.*, 1983; Bose and Davies, 1984). However, a minor lesion can often exert a major biological response as has become obvious for (6-4) photoproducts. Furthermore, through this current work, it has become clear that this adduct appears to be more prevalent than initially believed (~6% the frequency of cyclobutane dimers). Two other important conclusions were derived from this investigation. Firstly, TA^* photoproducts appear to be recognized and repaired by the same pathway as are (6-4) photoproducts. In normal human fibroblasts and fibroblast strains representing each of the eight XP complementation groups, the repair of TA^* adducts paralleled exactly (6-4) photoproduct removal. Thus, a partial defect in (6-4) photoproduct repair coincided with a partial defect, to the same degree and with identical kinetics, in TA^* lesion removal. Secondly, it is quite clear that XP variant cells were capable of repairing this photoproduct. This eliminates another photoproduct as the potential source of its UV-sensitive phenotype.

One of the primary motivational forces towards establishing an enzymatic hydrolysis approach to identify the UV-lesions contained in excision fragments was the need to better characterize and quantitate cyclobutane dimers with a severed intradimer phosphodiester bond. Previous work from our laboratory (Weinfeld *et al.*, 1986; Paterson *et al.*, 1987) had found evidence of such a modification in both excision fragments from normal human fibroblasts and in the genomic DNA of post-UV incubated XP group D cells. However, little more could be ascertained about these sites with the techniques then available. Using the enzymatic digestion / HPLC methodology described here, it became possible to illustrate that although a cleaved intradimer phosphodiester bond was the most common condition (~85%) for a cyclobutane dimer found in excision fragments from post-UV incubated normal human fibroblasts, this was not absolute, which argues against this severage being a prerequisite to incision by the excision repair complex as had originally been proposed (Paterson *et al.*, 1987). Furthermore, modified dimers were found (in approximately the same proportion) in excision fragments isolated from all cell types examined, as long as the particular cell type was capable of some cyclobutane dimer repair. This includes several XP fibroblasts strains, TTD strains, a CHO normal cell line, two UV-sensitive CHO cell lines, wild type *E. coli*, and numerous mutant *E. coli* strains. Thus, intradimer phosphodiester bond cleavage is a wide spread phenomenon and not peculiar to mammalian systems. Significantly, (6-4) photoproducts were not found modified in this manner. The necessity of this modification for cell survival following UV-irradiation became an intriguing consideration.

Since none of the UV-sensitive mammalian cell strains studied lacked intradimer phosphodiesterase activity, and since attempts to isolate the human IDP gene were facing typical difficulties, the genetically simpler organism, *Escherichia coli*, was chosen as an alternative system to gain some insight into the function of intradimer phosphodiester bond cleavage. Because of the numerous UV-sensitive *E. coli* mutants available, it seemed a reasonable hypothesis that one of these may harbor a defect in the IDP gene. To this end, genomic DNA and excision products isolated from several post-UV incubated mutant and wild-type strains were analyzed.

To summarize the results of this work, none of UV-sensitive mutants analyzed, which included *uvrA*, *umuC*, *recA*, *recBC*, and *lexA*, lacked the capacity to sever the interpyrimidine phosphodiester bond of a cyclobutane dimer. Furthermore, the proportion of excised dimers harboring this modification in the various mutants indicated that the IDP gene is not under SOS control. However, modified dimer sites could be found in post-UV incubated *uvrA* cells, adding support to the idea that intradimer phosphodiester cleavage is more than a post-excision event.

To date, the *in situ* study of intradimer phosphodiesterase activity has been informative but has not revealed the cellular function of this evolutionarily conserved activity. To better address this question, an *in vitro* system has been set up to explore the action of polymerases and repair enzymes upon encountering a conventional cyclobutane dimer versus its modified counterpart. Cyclobutane dimers, in their intact state, are well documented polymerase-blocking lesions in both prokaryotes and eukaryotes (Caillet-Fauquet *et al.*, 1977; Villani, G., *et al.* 1978; Moore *et al.*, 1981; Vos and Rommelaere, 1982; Griffiths and Ling, 1989; Selby and Sancar, 1990). However, in UV-sensitive cells, the cytotoxic effects of UV-irradiation more closely parallel the persistence of (6-4) photoproducts, not cyclobutane dimers (Cleaver *et al.*, 1988). This suggests that there exists some mechanism to cope with cyclobutane dimers which persist in genomic DNA. The discovery of modified cyclobutane dimers in the genome of post-UV incubated cells which were deficient in their ability to remove this class of UV damage [XP groups A and D (Paterson *et al.*, 1987), normal CHO cells (Pirsel *et al.*, 1989), *E. coli uvrA* strain (this study)], led to the premise that intradimer phosphodiester bond cleavage may alleviate helical distortion, thus allowing replication and/or transcription enzymes to bypass these otherwise polymerase-blocking lesions.

During this period of time, preferential repair of cyclobutane dimers from transcriptionally active sequences was described (Bohr *et al.*, 1985; Mellon *et al.*, 1986; Bohr *et al.*, 1987; Mellon *et al.*, 1987). The ability of cells to remove these lesions from essential sequences appeared to account for normal cell survival in the absence of general cyclobutane dimer repair. However, preliminary work in our laboratory revealed that modified dimers appeared transiently in transcriptionally active sequences of post-UV incubated normal human fibroblasts, and it was hypothesized that what had been monitored as repair in the studies on preferential repair, was actually modification. This theory required that the T4 endonuclease, DenV, which had been used to monitor repair in transcriptionally active genes, was not capable of recognizing a cyclobutane dimer with a severed intradimer phosphodiester bond. To address this and other questions in a more direct way, a covalently closed circular substrate was constructed which contained (i) no lesion, (ii) a cyclobutane dimers at a defined position within the *lacZ* gene, or (iii) a cyclobutane dimer, with its interpyrimidine phosphodiester linkage severed, at the same location. These have since been used in a series of preliminary *in vitro* experiments with a limited repertoire of enzymes. It is, however, clear that the endonuclease activity of DenV cannot incise the DNA at the position of a modified cyclobutane dimer. Furthermore, the viral SP6 RNA polymerase, while unable to bypass an intact cyclobutane dimer, appears to be able to continue past this lesion if the phosphodiester bond between the dimerized pyrimidines has been cleaved. In addition, these studies indicate that a cyclobutane dimer, whether modified or not, represents an absolute block to *E. coli* DNA polymerase I. Although the data on the action of DNA and RNA polymerases upon encountering an intact versus modified cyclobutane dimer are still preliminary, these results, combined with the inability of DenV to act on modified dimers, maintains a role for intradimer phosphodiesterase in bypass of cyclobutane dimers during transcription.

In conclusion, the enzymatic hydrolysis / HPLC methodology, which has been gainfully employed in this dissertation, offers a new approach to assess the repair capacity of a cell. This protocol has enabled confirmation and extension of the results of others on the repair of cyclobutane dimers and (6-4) photoproducts in normal and UV-sensitive cells of various origins. It has, furthermore, made it possible to follow the repair of the less frequently studied TA* photoproduct and the novel modified cyclobutane dimer. The accumulated data on cyclobutane dimers with an interpyrimidine phosphodiester cleavage in mammalian cells has encouraged further exploration of the cellular function of the associated enzyme. This has resulted in evidence for this activity operating in the bypass of these otherwise polymerase-blocking lesions, during transcription.

Although the work presented in this dissertation has added to our knowledge of the cellular processing of UV-damage, it has also served to open up several new avenues of research. It is felt that the enzymatic hydrolysis / HPLC protocol is the methodology of choice to assess the repair of UV-induced DNA damage. This procedure can, therefore, be used to ascertain the repair phenotype of other UV-sensitive cell strains which have not been previously defined, as well as to confirm and extend data on those which have been previously characterized. A representative of the human genetic disorder, Cockayne's syndrome, and an *E. coli* strain carrying the *mfd* mutation, command immediate attention. These mutants are known to be defective in preferential repair and are therefore ideal candidates for harboring a mutation in the IDP gene. If these were, in fact, IDP mutants, this would not only provide an alternative approach to gene cloning, but would provide additional information on the role of intradimer phosphodiesterase cleavage as it pertains to transcription past a cyclobutane dimer.

If, in fact, IDP functions in bypass of cyclobutane dimers during transcription, the only anticipated phenotype associated with a mutation in the IDP gene is deficient preferential repair, as is the case for the *mfd* mutation. This should translate into a UV-sensitivity. Thus, further exploration of UV-sensitive *E. coli* mutants may reveal an IDP mutant if the *mfd* gene fails to coincide with the IDP locus. If not, there are many well-defined UV-sensitive *Saccharomyces cerevisiae* mutants which may warrant evaluation of IDP function for the purpose of gene cloning, if such an activity exists in this species.

Although substrates have been constructed to test the action of polymerases and repair enzymes upon encountering an intact versus modified cyclobutane dimer, the various experiments performed to date only scratch the surface of the countless opportunities offered by this system. The most immediate focus will be the completion of *in vitro* replication and transcription analyses. DNA synthesis can be primed by use of a reverse-sequencing primer which binds upstream of the inserted lesion-containing oligonucleotide. Because replication is initiated in this manner, there is no requirement for replication control sequences and thus, *in vitro* addition of nucleotides to the 3'-OH of the reverse-sequencing primer can utilize any DNA polymerase (including *E. coli* polymerase I, *E. coli* polymerase III, and mammalian polymerases) made available for DNA synthesis. Although preliminary results have indicated that *E. coli* polymerase I cannot bypass a modified dimer, this may not be characteristic of an S-phase DNA polymerase such as *E. coli* Pol III. The activity of other prokaryotic and eukaryotic polymerases are therefore worth assessing despite the result obtained with *E. coli* Pol I. Alternatively, it may be necessary to add crude cellular extract to the *in vitro* replication mix, as some factor necessary for bypassing a modified dimer may be lacking in this defined *in vitro* system. On the other hand, failure to detect *in vitro* bypass may reflect the lack of natural DNA topology. This may be overcome by supercoiling the substrate molecule, through the use of *E. coli* gyrase, prior to *in vitro* replication studies.

The available control sequences in the pGEM vector render the dimer-containing construct most readily amenable to *in vitro* transcription by the T7 and SP6 viral RNA polymerases. Because of the strand-specific nature of a cyclobutane dimer and the orientation with which the constructs were made, initiation from the SP6 start site places the lesion on the transcribed strand, whereas initiation from the T7 start site places the dimer on the non-transcribed strand. Following confirmation of the results achieved to date with SP6 RNA polymerase, it will be important to illustrate that *E. coli* RNA polymerase behaves in the same manner. *In vitro* initiation from the *lacZ* gene, in the presence of c-AMP and c-AMP receptor protein, will place the lesion in the proper orientation. To extend the *in vitro* work to mammalian RNA polymerases, specific control sequences will need to be inserted into the pGEM vector.

It is well documented that two different repair enzymes, although specific for cyclobutane dimers, exhibit no or decreased activity towards a dimer with a severed interpyrimidine phosphodiester bond. For example, as demonstrated in this dissertation, the T4 UV endonuclease was unable to incise the DNA at the sight of a modified cyclobutane dimer even given an amount of enzyme 80-fold in excess of that required to act on a conventional dimer. In addition, *E. coli* photolyase, while able to photoenzymatically reverse the cyclobutyl ring adjoining two pyrimidines which lack a phosphodiester linkage, requires an approximate 10-fold excess of enzyme to achieve the same reaction efficiency enjoyed by a cyclobutane dimer with its phosphodiester bond intact (Liuzzi and Paterson, in press). It is, therefore, of interest to ascertain the activity of other repair enzymes on these two types of dimers, most notably the UvrABC complex. As a cyclobutane dimer is not the best substrate for this nucleotide excision repair enzyme (Sancar *et al.*, 1984), it will be of interest to determine whether modification enhances or diminishes recognition. Similarly, given the recent development of an *in vitro* human nucleotide excision repair system which appears to act almost exclusively on (6-4) photoproducts (Jones *et al.*, 1992), the possibility exists that dimer modification is also beneficial for recognition and incision by mammalian repair enzymes.

Since pGEM vectors harbor many control sequences for *in vivo* studies in *E. coli*, repair and recombination-deficient strains (eg. *uvrA recA* mutant) can be transformed with that cyclobutane dimer-containing construct which possesses a normal intradimer phosphodiester linkage. Although the lesion would be diluted during replication, if only limited DNA synthesis was allowed (by, for example, utilizing a *polC*^{ts} host), the vector DNA could be re-isolated from the host cells and subsequent analysis would still allow adequate detection of lesions. If synthesis was carried out in the presence of bromouridine, the phosphodiester bond status of a cyclobutane dimer which was replicated across could be assessed and compared to one in unreplicated DNA. Along the same line, the status of the cyclobutane dimer in the presence and absence of transcription could be compared by transforming a Δ*lacZ* host, and culturing in the presence or absence of IPTG. This inducer would execute transcription of the *lac* gene which is present on the pGEM vector. As the lesion-containing oligonucleotide has been inserted within the *lacZ* transcriptional unit, this would provide an *in vivo* test of the ability of RNA polymerase to continue past a cyclobutane dimer (which it cannot perform *in vitro*) by monitoring for gene expression. The status of a bypassed dimer could then be assessed. Parallel experimentation could be performed in mammalian cells but would require the insertion of both control sequences for maintenance of the vector in these cells and an appropriate transcription unit.

Although it appears that IDP cannot sever the interpyrimidine phosphodiester bond of a (6-4) photoproduct, the *in vitro* and *in vivo* assessment of the action of replication and transcription polymerases upon encountering this lesion, or alternatively, a TA* photoproduct, is of interest. Construction of suitable substrates can proceed along similar lines to what has been done for the cyclobutane dimer-containing constructs. *In vivo* studies may reveal a yet unknown modification which relieves a block to the progression of polymerases generated by either or both of these lesions. Vectors containing one specific class of UV photoproducts will also allow the assessment of the relative activity of various repair enzyme on the different adducts, which could be of great value in understanding how repair of these lesions is affected in repair-deficient cells such as xeroderma pigmentosum strains.

In conclusion, the work which has been described in this dissertation has hopefully expanded our understanding of the processing of UV-induced DNA damage in both prokaryotic and mammalian cells. As is true of most research, it has also uncovered many new questions. It is hoped that through the continuation of the work which has been initiated here, we will come to recognize and understand the complexity of the ways in which cells cope with and overcome damage to the most central part of their existence, their DNA.

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APPENDICES

APPENDIX A - DETAILED METHODOLOGIES UTILIZED IN IDENTIFICATION AND QUANTITATION OF UV PHOTOPRODUCTS INDUCED IN AND EXCISED FROM THE GENOMIC DNA OF LIVING CELLS

All chemicals used in the experiments detailed in this dissertation, unless specifically stated otherwise, were purchased from BDH Chemicals (Toronto, ON). All glassware was Pyrex brand and was obtained from Corning Incorporated (Corning, NY). Tissue culture and media supplies, unless otherwise noted, were obtained from GIBCO/BRL Inc. (Burlington, ON). The source of all other general laboratory supplies, unless otherwise stated, was Fischer Scientific (Pittsburg, PA). Double-distilled H_2O was purified through a Nanopure II filtering system (Barnstead/Sybron, Boston, MA).

I. Culturing of Human and Chinese Hamster Ovary Cell Lines The mammalian cell strains used in the various studies include: i) a fibroblast strain established from a skin biopsy of a disease-free human subject; ii) several fibroblast strains established from skin biopsies of human patients who were afflicted with xeroderma pigmentosum, trichothiodystrophy, or an uncharacterized UV-sensitivity; iii) a repair-proficient cell line established from two subsequent subclonings of a culture derived from Chinese hamster ovarian cells, and selected on the basis of colony morphology in soft agar and resistance to 8 μg/ml 8-azaadenine; and iv) two mutant Chinese hamster ovary cell lines, mutagenized by ethyl methanesulfonate treatment and isolated on the basis of their enhanced sensitivity to UV radiation. The normal human fibroblast strain (encoded **GM38**; 9-year-old black female), along with two of the eight xeroderma pigmentosum fibroblast cell strains [**GM3542** (XP group F; 29-year-old Japanese male with no neurological abnormalities) and **GM3021** (XP group G; 17-year-old female with neurological abnormalities and acute sun sensitivity)], were purchased from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). The remaining XP fibroblast strains [**CRL1223** (XP group A; 7 year-old female with neurological complications; formerly XP12BE), **CRL1199** (XP group B; no patient information available), **CRL1166** (XP group C; no patient information available; formerly XP2BE), **CRL1157** (XP group D; 19-year-old female; formerly XP6BE), **CRL1259** (XP group E; 34-year-old female; formerly XP2RO), and **CRL1162** (XP variant; 27-year-old male; formerly XP4BE)] were obtained from the American Type Culture Collection (Rockville, MD). All three trichothiodystrophy fibroblast strains, **TTD1GL** (class 1; 4-year-old white male not displaying photosensitivity), **TTD2GL** (class 2; 2-year-old white female not displaying photosensitivity), and **TTD1B1** (class 3; 6-year-old male with no photosensitivity) were supplied by Dr. Alan Lehmann (Sussex University, Brighton, UK). The two remaining human fibroblast strains, **BD-1** (4-year-old white female exhibiting hypo- and hyperpigmentation on sun-exposed areas of the skin) and **CVT** (6-year-old white male exhibiting an unusual 'rash' on sun-exposed area), were established from skin biopsies of patients showing UV-sensitivities of unknown origins. **BD-1** was provided by Dr. R. Ramssey (Toronto Hospital for Sick Children, ON). **CVT** was obtained from Dr. Betsy Hirsch (University of Minnesota, Minneapolis, MN). The repair proficient CHO cell line, **AA8**, and its two UV-sensitive derivatives, **UV-4** and **UV-5** were gifts from Dr. Larry H. Thompson (Lawrence Livermore Laboratory, Livermore, CA). Cultures received from these various sources were expanded, harvested, and frozen in aliquots of ~ 10^6 cells, which were subsequently stored in liquid nitrogen freezers.

For human fibroblast strain, the lowest passage numbers available were secured for further study. This varied with cell strain, and corresponded to the following: **GM38**: passage 13; **CRL1223**: passage 11; **CRL1199**: passage 14; **CRL1166**: passage 12; **CRL1157**: passage 13; **CRL1259**: passage 14; **GM3542**: passage 14; **GM3021**: passage 12; **CRL1162**: passage 11; **TTD1B1**: passage 12; **TTD1GL**: passage 14; **TTD2GL**: passage 14; **BD-1**: passage 9; and **CVT**: passage 11. As the CHO cell lines used in this study have been immortalized, they do not have passage numbers.

All tissue culture manipulations were performed in a laminar flow cabinet (NuAire, Inc., Plymouth, MW). Cells were retrieved from the liquid nitrogen freezers, thawed, and added to 20 ml of prewarmed (37°C) Ham's F12 medium [which had been supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin G, and 100μg/ml streptomycin sulfate] in a 150-mm tissue culture dish (Lux Scientific Corp., Newbury, CA). Ham's F12 media was purchased as a dry nutrient mixture (containing *L*-glutamine but lacking sodium bicarbonate). This was dissolved in the appropriate quantity of ddH_20, supplemented with 1.176 g $NaHCO_3/l$ of medium, and the pH adjusted to 7.0 with HCl. This was then sterilized through a positive-pressure filter, and stored in sterile bottles for later use. Fetal calf serum, glutamine, penicillin G, and streptomycin were added to this preprepared media immediately prior to usage.

Cultures were incubated at 37°C in a humidified (75-85%) atmosphere of 5% CO_2-95% air (Lunaire Incubator, Lunaire Environmental Incorporated, Williamsport, PA). Cells were grown to confluence, which normally required 9-10 days for cells which had been previously frozen.

During this time, medium was routinely changed (every three days) by syphoning the liquid from the tissue culture plate and adding 20 ml of fresh prewarmed medium. After reaching confluence, cells were reseeded into four new 150-mm dishes for human fibroblasts strains, or ten of the same dishes for CHO cell lines. To accomplish this, medium was first removed from the cells. Each plate was then washed twice with room temperature PBS (0.2 M NaH_2PO_4/Na_2HPO_4 (pH 7.2)], and supplemented with 1 ml of a 1% trypsin/1 mM EDTA (ultraPURE, BRL) solution. After incubating for 2-3 min at 37°C in the tissue culture incubator, cells were collected by resuspending the detached cells with 5 ml of medium. One and a half ml of the cell suspension was then distributed to each of 4 tissue culture plates for human fibroblasts strains, or 0.5 ml to each of 10 dishes for CHO lines. Prewarmed medium was added to each dish to bring the final volume up to 20 ml. Cells were again grown to confluence, with regular intervening media changes. In the case of human fibroblasts, this cycle was repeated to generate a total of 16 confluent dishes. The cells were then harvested from all dishes, collected by centrifugation, washed twice with ice-cold PBS, and resuspended with 10 ml of cold PBS. For CHO cells, 10 confluent dishes was sufficient for further experimentation. Resuspended cells were stored in a 15-ml polystyrene tube (Corning Incorporated) on ice for subsequent experimental use.

Cell concentration of the harvested cells was determined by diluting 0.2 ml of a cell suspension into 9.8 ml of PBS, in a clear plastic container. Cell count measurements were taken in a Coulter Counter (Model ZM, Coulter Electronics of Canada Ltd., Burlington, ON), and the concentration of the original suspension was subsequently calculated.

II. Culturing of *E. coli* *The E. coli* strains employed in this investigation include i): two wild-type strains with different genetic backgrounds {**AB1157** [xyl-5, mtl-1, galK2, λ-rac$^-$, rpsL31, kdgk51, Δ(gpt-proA)62, lacY1, tsx-33, supE44, thi-1, leuB6, hisG4, mgl-51, arg-3, rfbD1, ara-14, thr-1] and **JC9937** [thi-1, leu-6, proA1, his-4, argE3, mtl-1, lacY1, galK2, ara14, xyl-5, srlD, str31 (SmR), tsx-33]}; ii) several UV-sensitive mutant strains {**TK603** (AB1157 arg$^+$, ilv^{ts}, uvrA6), **TK610** (AB1157 arg$^+$, ilv^{ts}, uvrA6, umuC36), **GW3198** (AB1157 umuC36), **GW2727** [AB1157 lexA3(Ind$^-$), malE::Tn10], and **SMR374** [JC9937 Δ(srlR-recA)306::Tn10]}; and iii) a recombination-deficient mutant strain [**DL184** (AB1157 recB21, recC22)]. **AB1157**, **TK603**, **TK610**, **GW3198**, and **GW2727** were supplied by Dr. Graham Walker (Massachusetts Institute of Technology, Cambridge, MA) and were received as glycerol stocks. **DL184**, **JC9937**, and **SMR374** were gifts from Dr. Susan Rosenberg (University of Alberta, Edmonton, AB) and were acquired as single-colonies growing on agar plates.

M9CA medium was used in the culturing of all *E. coli* strains. Liquid medium consisted of 1.3% $Na_2HPO_4 \cdot H_2O$, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 2 mM $MgSO_4$, 0.2% glucose, 100 µM $CaCl_2$, and 0.2% casamino acids (Difco Laboratories, Detroit, MI). In the preparation of liquid medium, 12.8 g $Na_2HPO_4 \cdot 7H_2O$, 3 g KH_2PO_4, 0.05 NaCl, and 1 g NH_4Cl were dissolved in 988 ml of ddH_2O in a 2-*l* Erlenmeyer flask, and sterilized by autoclaving for 15 min at 15 lb/sq. in. on a liquid cycle. After cooling to ~50°C, this salt solution was supplemented with 10 ml of a 20% filter sterilized (115-ml Nalgene disposable filter unit; cellulose nitrate membrane; 0.45 µm pore size; Nalge Company, Rochester, NY) glucose solution, 10 ml of a 20% filter sterilized casamino acids solution, 1 ml of sterile 2 M $MgSO_4$, and 1 ml of sterile 0.1 M $CaCl_2$. For agarose plates, 15 g/*l* of bacto-agar (Difco Laboratories) was added to the salt solution prior to autoclaving. After addition of the remaining nutrients, the medium was poured (30-35 ml/plate) into 90-mm bacterial culture plates (Fisher Scientific).

For those strains received as glycerol stocks, M9CA agar plates, supplemented with 1 µg/ml thiamine (Sigma Chemical Company, St. Louis, MO), were streaked for single colony growth and incubated at 37°C overnight, inverted, in a bacterial incubator (model 8597; Lab-Line Instruments, Inc., Melrose Park, IL). The following afternoon, a single colony was used to inoculate 10 ml of M9CA (also supplemented with 1 µg/ml thiamine) in a 20 x 150-mm disposable borosilicate glass culture tube (Fischer Scientific). This overnight culture was incubated at 37°C, with vigorous shaking.

III. Introduction of Labelled Nucleotides and UV Adducts into Cellular DNA of Mammalian Cells

A. Radioactive Labelling of Mammalian Cellular DNA For each cell line, approximately 2.5×10^6 cells were seeded in each of thirty-six 150-mm plastic tissue culture dishes and incubated overnight in ~20 ml of Thy-free Ham's F12 medium (supplemented as above) to establish exponential growth. In the morning, this medium was removed and replaced with 20 ml of the same medium containing, in addition, 5 µCi/ml [^3H] dT (1 mCi/ml; 41 Ci/mmol; NEN Canada, Montreal, PQ). These cultures were incubated for an additional 48 hr in the case of human fibroblasts, or 24 hr for CHO cell lines. The radioactive medium was then removed, and each monolayer culture was subsequently washed with 10 ml of PBS. Fresh nonradioactive Ham's F12 medium was added and cultures were incubated for 24 hr to deplete endogenous precursor pools of tritium label. Because of the radioactive nature of all media and buffers used in association with [^{3}H]-labelling and subsequent steps, their removal from the monolayers involved careful pipetting and disposal.

B. UV-Irradiation and Subsequent Incubation of Mammalian Cell Cultures After removal of the post-labelling medium from each dish, cultures were washed once with PBS and drained thoroughly. Thirty out of the 36 dishes of radioactively labelled fibroblasts were immediately exposed, in open dishes and one plate at a time, to a UV fluence of 40 J/m^2 delivered by two 15-W germicidal (low-pressure mercury vapor) lamps (Model GE 15T8; General Electric, Toronto, ON), and emitting 97% of their radiant energy at 254-nm wavelength. The incident fluence rate of the UV source was 2.3 W/m^2, as measured with a UVS Radiometer (UVP Inc., San Gabriel, CA). Immediately following exposure, each dish was replenished with fresh Ham's F12 growth medium. The remaining 6 dishes of cells were sham exposed, thereby serving as unirradiated controls. In this case, following washing with PBS, the growth medium was simply replaced without further manipulation. Six dishes of UV-irradiated cells were harvested immediately, 1 for obtaining genomic DNA and the remaining 5 for collecting the TCA-soluble material, as described below. The remaining 24 UV-exposed and 6 sham-irradiated dishes were incubated, as described above, to allow the cellular machinery time to repair (or to attempt to repair) the UV-induced DNA damage. Six of the remaining 24 dishes of UV-irradiated cells (1 for genomic DNA, 5 for TCA-soluble material) were harvested after a post-UV incubation period of each of the following times: 3, 6, 12, and 24 hr. The cells which had been sham-irradiated dishes were harvested after 24 hr for human cells, or 12 hr for CHO cells.

IV. Introduction of Labelled Nucleotides and UV Adducts into Genomic DNA of *Escherichia coli*

A. Radioactive Labelling of Bacterial Cellular DNA In duplicate, 5 ml of an overnight culture were each added to 200 ml of fresh M9CA medium, supplemented with thiamine and containing, in addition, 5 μCi/ml [^{3}H]-thymidine (1 Ci/ml; 58 mCi/ml; NEN Canada, Montreal, PQ) and 200 μg/ml 2-deoxyadenosine. The presence of 2-deoxyadenosine in the medium allows the uptake and incorporation of thymidine into the DNA of a nonthymidine-requiring cell. These cultures were incubated in 1-*l* flasks at 37°C for ~2 hr (with vigorous shaking) to re-establish logarithmic growth, and subsequently allow approximately 2 cell doublings. Incubation was continued until the OD_{600}, as monitored using a 1-cm disposable styrene cuvette (Fischer Scientific) in a LKB Novaspec spectrometer (LKB Biochrom, Cambridge, England), had reached ~0.3. Cells were then collected by centrifugation at 6000*g* for 10 min at 4°C, using a HB-4 rotor in a Sorvall RC-5C Refrigerated SuperSpeed Centrifuge (DuPont Canada, Inc., Mississauga, ON). The cell pellets from both 200-ml cultures were washed once with ice-cold NT buffer, polled, and resuspended in a total of 200 ml of the same buffer. This cell suspension was stored on ice until further use.

NT buffer [0.1 M NaCl, 10mM Tris-HCl (pH 7.4)] was made by dissolving 5.8 g of NaCl in 900 ml of ddH_20, adding 10 ml of a 1 M Tris-HCl (pH 7.4) solution, adjusting the volume of the buffer to 1 *l*, and finally autoclaving to sterilize the solution. The 1 M Tris-HCl stock buffer was made by dissolving 121.1 g of Tris base in 800 ml of ddH_20 and adjusting the pH to 7.4 with HCl (approximately 70 ml). The volume was then adjusted to 1 *l* and sterilized by autoclaving.

B. UV-Irradiation and Subsequent Incubation of Bacterial Cultures For the wild-type strain, **AB1157**, a 20-ml aliquot of resuspended cells was placed in a 150-mm plastic tissue culture plate (Lux Scientific Corp.) and irradiated, with continual mixing, at a fluence of 40 J/m^2. (Lamp source and specifications are described above). For the 0 min post-UV incubation time point (i.e. no repair), these UV-treated cells were placed immediately on ice, and kept cold until the TCA-soluble material could be isolated (see below for details of this procedure). Each successive 20-ml aliquot (8 aliquots in total) of irradiated cells was added directly to 200 ml of prewarmed (37°C) thiamine-containing M9CA (no radiolabel present). Cultures were then incubated in 1-*l* flasks in the dark (to inhibit photoreactivation) at 37°C, with vigorous shaking. Each cell culture was harvested at a defined post-UV incubation time (5, 15, 30, 45, 60, 90, or 120 min). A 20-ml aliquot of resuspended cells was subject to the same set of manipulations, but not exposed to UV light (unirradiated control). Following sham-exposure, these cells were similarly added to M9CA + thiamine medium and incubated for 120 min prior to harvesting.

For the remaining strains, cells were grown as for **AB1157**, but only 2-20 ml aliquots were irradiated. One of these was placed on ice immediately following UV-treatment and served as the unrepaired control. The second fraction was incubated for 120 min in M9CA + thiamine following UV-irradiation as described above. Again, a third 20-ml portion sham-irradiated and subsequently incubated as described above for **AB1157**.

V. **Collection of High-Molecular-Weight DNA from Mammalian Cells** Following the appropriate combination of UV-irradiation and post-UV incubation, one 150-mm plate per experimental condition of human fibroblasts or CHO cells, were harvested by trypsinization for the isolation of genomic DNA. This procedure required removal of the culture medium by pipetting (as it still had some radioactive content), 2 successive washes of the cells with PBS, and incubation at 37°C for 2 min after the addition of 1 ml of a 1% trypsin/1 mM EDTA solution. After detachment of the cells from the tissue culture plate, they were resuspended with 6 ml of Ham's F-12 medium (to inhibit the action of trypsin) and immediately placed on ice in a 50-ml polypropylene tube (BlueMax 2098, Becton Dickinson Labware, Lincoln Park NJ). Cells were then collected by centrifugation at 1500*g* for 4 min at 4°C in a bench-top centrifuge (Accu-Spin FR, Beckman Instruments, Toronto, ON). Cells were subsequently washed twice with an equal volume of PBS, and repelleted in the same manner. The supernatant was removed, and the cells were resuspended in 10 ml of buffer containing 10 mM Tris-HCl (pH 7.4) and 0.1 M EDTA. This suspension was supplemented with 500 µl of a 10% (w/v) SDS solution (ultraPURE, BRL/GIBCO, Burlington, ON) and proteinaseK (20mg/ml; Boehringer Mannheim Canada, Dorval, PQ) to a final concentration of 100 µg/ml, and the lysed cell mixture was subsequently incubated overnight at 37°C. In the morning, the high-molecular-weight DNA was isolated by phenol/chloroform extraction. This procedure was executed as outlined below.

The sample of protease digested lysed cells was supplemented with an equal volume of redistilled phenol [which had been equilibrated with 0.1 M Tris-HCl (pH 8.0)], and mixed by inversion to form an emulsion. The phases were then separated by centrifugation at 1600*g* at room temperature for 5 min (Beckman Bench-top centrifuge). The aqueous (top) phase was transferred to a fresh 50-ml polypropylene tube and the extraction procedure was repeated once with an equal volume of a 1:1 mixture of phenol and chloroform (containing 4% v/v isoamyl alcohol), and for a third time with an equal volume of chloroform/isoamyl alcohol. After transferring the aqueous phase for a final time to a fresh polypropylene tube, the DNA was precipitated by the addition of 0.1 volume of 2.5 M sodium acetate (pH 5.2) and 2 volumes of ice-cold 100% ethanol. Following mixing, the contents were stored overnight at -20°C to allow the DNA precipitate to form. In the morning, the DNA was recovered by centrifuging at 12,000g for 30 min at 4°C (HB-4 rotor; Sorvall RC-5C Refrigerated SuperSpeed Centrifuge), and dried, under vacuum, in a SpeedVac Concentrator (model SVC 100H; Savant Instruments, Inc., Farmingdale, NY). The nucleic acid pellet was subsequently resuspended in 10 ml of 10 mM Tris (pH 7.4),and supplemented with 10 µl of 1 M $MgCl_2$ and 50 µl of a 10 mg/ml RNase A (Boehringer Mannheim Canada) solution [dissolved in 10 mM Tris-Cl (pH 7.5) 15 mM NaCl and heated to 100°C for 15 min to remove any contaminating DNase]. Following incubation for 1 hr at 37°C, 500 µl of 10% SDS and 25 µl of a 20 mg/ml proteinaseK solution were added to the mixture, and incubation was continued overnight. The following morning, the DNA was purified by extraction with phenol/chloroform and subsequent overnight ethanol precipitation, as described above. After recovery of the DNA by centrifugation, it was dried and resuspended in 1 ml of ddH_2O. The specific activity of the purified DNA was calculated by determining both the total amount of DNA and radioactivity present. DNA concentration was determined by measuring the OD_{260} (Lambda 4B UV/VIS Spectrophotometer, Perkin-Elmer, Sherwood Park, AB) of the entire DNA sample in a 1 cm-wide quartz glass cuvette (Hellma Canada Limited, Concord, ON). Assuming 1 OD_{260}/cm corresponds to 50 µg/ml of double-stranded DNA, the concentration, and therefore the total amount of DNA present was computed. To ascertain the total radioactive content, a 10-i sample was mixed with 0.1 ml of H_2O and 5 ml of EP scintillation cocktail (Beckman). The radioactive content of this sample was measured in a liquid scintillation system (Beckman model LS 5801 liquid scintillation spectrometer). This purified DNA had a specific activity ranging from ~280,000 - 960,000 dpm/µg.

VI. Collection of High-Molecular-Weight DNA from *E. coli* The 200-ml bacterial cultures of strains **TK603** and **TK610**, were harvested by centrifugation at 4000*g* for 10 min at 4°C (HB-4 rotor; Sorvall RC-5C Centrifuge), washed twice with NT buffer, and repelleted under the same conditions. Cells were then resuspended in 50-ml polypropylene tubes in 2 ml of lysis buffer [containing 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5% glycerol (v/v), and 300 µg/ml lysozyme

(Sigma Chemical Company)], and incubated on ice for 1 hr. Cell lysis was completed by the addition of 0.05% Nonident-40 (v/v; Sigma Chemical Company). Cellular debris was removed by centrifugation (10,000*g* for 30 min at 4°C; Sorvall RC-5C Refrigerated SuperSpeed Centrifuge). The supernatant was retained, and the nucleic acids subsequently purified by phenol/chloroform extraction and overnight ethanol precipitation in the same manner as described above for mammalian cells. The pellet was then collected by centrifugation (15,000*g* for 15 min at 4°C; Sorvall RC-5C Refrigerated SuperSpeed Centrifuge), taken to dryness, and resuspended in 2 ml of ddH_20. Specific activity of the radiolabelled genomic DNA was determined in a manner analogous to that employed for mammalian cells, and ranged from 300,000 - 620,000 dpm/µg.

VII. Collection of Excision Fragments from Mammalian Cells and *E. coli* Following the appropriate combination of UV-irradiation and post-UV incubation, five 150-mm dishes per experimental condition of mammalian cells, or 200 ml of bacterial culture (all strains except **TK603** and **TK610**), were harvested and washed in the same manner as described for the isolation of high-molecular-weight DNA. Each cell pellet was resuspended, to a total volume of 0.5 ml, in 0.1 M Tris-HCl (pH 7.5), and transferred to 1.5-ml polyurethane tubes (Eppendorf, Brinkmann, Rexdale, ON). Each of two 10-µl samples (of each cell suspension) was added to 0.1 ml of H_2O, lysed by the addition of 0.1 ml 10% TCA, and mixed with 5 ml of EP scintillation cocktail. The radioactive content of the samples were measured in a liquid scintillation system. These values were used to calculate the total radioactivity of the cell samples. The remainder of each cell suspension was added to 0.5 ml of 10% TCA, and mixed thoroughly by vortexing (Type 37600 mixer, Thermolyne, Sybron/Brinkmann, Rexdale, ON). The resultant cell lysate was held on ice for 15 min. The TCA-soluble and -insoluble fractions were separated by centrifugation (10,000*g* for 15 min at 4°C) in a microfuge (Eppendorf Centrifuge 5415, Brinkmann, Rexdale, ON). The acid-soluble material (~900 ul) was reserved and transferred to a new 1.5-ml polyurethane tube. The total radioactivity in each acid-soluble fraction was calculated from the radioactive content of two 20-µl samples.

The remaining TCA-soluble fractions were extracted with diethyl ether to remove the TCA. Samples were transferred to 15-ml polypropylene tubes (Becton Dickinson Labware) and supplemented with 5 ml of diethyl ether which had been stored at 4°C. The contents were mixed by inversion, and subsequently left standing at room temperature for 5 min in order for separations of the phases. The organic (top) phase was then removed, and the process repeated with fresh ether. Extraction was carried out 5 times in all. The removal of TCA from the aqueous phase was ascertained by the return to neutral pH. Each DNA solution was then taken to dryness under vacuum and resuspended in 0.3 ml of ddH_2O. All samples were stored at -20°C until analyzed.

VIII. Quantitation of UV Adducts in Genomic DNA

A. Primary Enzymatic Hydrolysis For each DNA sample, 5 samples of 10 - 20 µg of DNA (15 - 40 x 10^6 dpm) were each incubated overnight at 37°C (in a Lauda M20 Circulating Water Bath, Sybron/Brinkmann, Rexdale, ON), in 1.5-ml polyurethane tubes, in 1-ml reactions containing 20 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 2 mM $CaCl_2$, 150 units of staphylococcal nuclease (Boehringer Mannheim Canada), 200 units of DNase I (Boehringer Mannheim Canada), 0.025 units of snake venom phosphodiesterase (Sigma Chemical Company), and 20 units of calf alkaline phosphatase (Boehringer Mannheim Canada). This preliminary hydrolysis served to reduce the high-molecular-weight DNA to a series of lesion-containing trinucleotides (for DNA samples derived from UV-irradiated cells) plus mononucleosides.

B. Dialysis of Nucleic Acids

a) Dialysis of Small Molecules The samples of enzymatically hydrolysed DNA, described above, contain a preponderance of mononucleosides. This renders direct analysis of UV lesions present in such a sample impractical without first selectively reducing the radioactivity associated with nonlesion-containing molecules. As a potential means of satisfying this requirement, the usefulness of dialysis was investigated for selective removal of free bases and mononucleosides.

Approximately ~200,000 dpm of [^3H]-labelled thymine (48 Ci/mmol; NEN Canada, Montreal, PQ) was diluted with ddH_2O to a final volume of 1 ml. Two 10-µl aliquots were each added to 0.1 ml of ddH_2O and 0.5 ml of scintillation cocktail, and the radioactive content of each was determined. These values were used to calculate the radioactive content of the original sample. Dialysis tubing (3 kDa MW cut-off, 1 cm-wide; Spectra/Por, Fischer Scientific, Pittsburg, PA) was hydrated and cleaned by boiling (4-5 strips, 12 cm in length) in 1 *l* of ddH_2O for 15 min, followed by three successive washes with fresh ddH_2O. The remaining [^{3}H]-labelled thymine was placed inside a dialysis bag, the ends sealed with clips (Spectrum Medical Industries, BRL), and subsequently placed inside a 2-*l* beaker of ddH_20, and allowed to mix gently (using a metal stir bar; Nuova II Stir Plate) at room temperature. Two-10 μl samples were taken from the tubing at each of 1, 2, 3, 6, 12 and 24 hr from the onset of dialysis and their radioactive content was used to calculate the total radioactivity remaining in the dialysis tubing. During this 24-hr dialysis period, the 2 *l* of ddH_2O, which acted as a sink for molecules not retained by the dialysis tubing, was changed at 6, 12, and 18 hr.

This experiment was repeated using [^{3}H]-labelled thymidine (41 Ci/mmol; NEN Canada), the [^{3}H]-labelled cyclobutane dimer-containing dithymidylate, d-T<p>T, and the [^{3}H]-labelled cyclobutane dimer-containing trithymidylate, d-TpT<p>T. The latter two molecules were donated by Dr. Michel Liuzzi. Both had been isolated during the analysis of the action of snake venom phosphodiesterase on UV-irradiated [^{3}H] Thy-labelled poly(dA)·poly(dT) (Liuzzi *et al.*, 1989).

From this study, it was clear that dialysis could be employed to selectively reduce the vast excess of [^{3}H]-labelled thymidine from a sample of enzymatically hydrolysed high-molecular-weight DNA.

b) **Dialysis of Hydrolysed DNA Samples** Each set of five 1.5-ml reactions, representing enzymatic hydrolysis of a specific [^{3}H] thymidine-labelled genomic DNA sample, were pooled and the total radioactive content was ascertained. This was accomplished by measuring the radioactivity present in two 10-μl portions and calculating the radioactive content of the entire sample. The remainder of each sample was then dialysed, one sample at a time. As was done for the small [^{3}H]-labelled molecules described above, dialysis was continued for 24 hr for mammalian cells, or 12 hr for *E. coli*, against 2 *l* of ddH_20, with H_20 changes every 6 hr. The total radioactivity remaining in each sample was then established, in a manner analogous to the initial determination, and expressed as a percentage of initial radioactive content.

C. **DEAE-Cellulose Purification** For the analysis of cyclobutane dimers and (6-4) photoproducts, dialysis of the enzymatically hydrolysed DNA samples reduced the amount of [^{3}H]-labelled thymidine sufficiently to permit characterization and quantitation of these photolesions. However, for the study of TA* photoproducts, because under the chromatographical conditions employed this species elutes immediately following thymidine, a subsequent enrichment step was necessary to selectively reduce thymidine levels further. Therefore, for experiments involving analysis of TA* adducts, the material retained in the dialysis membrane was subjected to DEAE-cellulose chromatography.

Each dialysed hydrolysate was dealt with in the following manner. A 2-ml DEAE-cellulose column (Whatman DE 23, Whatman Inc., Clifton, NJ) was first equilibrated with 10 mM ammonium acetate (pH 7.0). The dialysed material, after determination of its radioactive content, (~1 ml) was loaded onto the column at a flow rate of ~10 ml/hr. The DEAE-cellulose matrix was subsequently washed with 10 ml of equilibration buffer (applied at approximately the same flow rate). As mononucleosides are not charged, and therefore not retained by the matrix, they will pass through the column at this step. The effluent fractions up to this stage were reserved in case later analysis suggested that charged species had not been retained on the column. Ten ml of elution buffer [0.4 M ammonium acetate (pH 7.0)] were then applied to the column, at the same flow rate, and the eluting material was collected in ten 1-ml fractions. Those fractions eluting with high salt and containing radioactivity (determined by measuring the radioactivity of a 10 µl aliquot of each fraction) were pooled, taken to dryness (under vacuum in a SpeedVac Concentrator), and redissolved in 1 ml ddH$_2$O. After determining the radioactive content of this trinucleotide-containing fraction, it was desalted using a Waters SEP-PAK C$_{18}$ cartridge (Waters Associates, Mississauga, ON) as outlined below.

A SEP-PAK cartridge was attached to the end of a plastic 5-ml syringe. The cartridge was first hydrated by passing 2 ml of 100% methanol though it. After washing 5 times with 5 ml of ddH$_2$O, the sample was applied to the column and the liquid was allowed to flow through under gravity. The cartridge was then washed with ddH$_2$O. The refractive index (Refractometer, Fischer Scientific Company) of each 1-ml fraction passing through the cartridge at this stage was ascertained and compared to pure ddH$_2$O (η = 1.3330). Once the refractive index had reached 1.3330, the column was washed with 1 additional ml of ddH$_2$O. The radioactive content of these fractions was also measured to ensure that radioactive molecules were not passing through the column. Having removed the salt from the trinucleotide preparation, 5 ml of 50% methanol (HPLC grade)/50% ddH_2O was applied to the column, and five 1-ml fractions were collected. Those fractions containing radioactivity were pooled, taken to dryness, and resuspended in 1 ml of ddH_2O.

D. Basic Three-Stage Analysis Procedure

a) **Formic Acid Hydrolysis** A 5,000 - 10,000 dpm sample of each trinucleotide-enriched SVP/CAP hydrolysate was added to 1 ml of 88% formic acid (BDH Chemicals, Toronto, ON) and transferred to a thick-walled (2mm) Pyrex tube. This tube was sealed using an acetylene torch, and subsequently placed in a 200°C oven (IsoTempOven Model 350G, Fischer Scientific) for 60 min. As the contents are under pressure following this procedure, the tube was first allowed to cool to room temperature, and subsequently placed in liquid nitrogen. Once the pressure inside the container had been reduced, it was opened using glass cutters and the contents were transferred to a 1.5-ml polypropylene tube. The formic acid was removed by evaporation in a SpeedVac Concentrator and the hydrolysate redissolved in 200 µl of ddH_2O prior to analysis by reverse-phase HPLC (as outlined below).

b) **CAP / NP1 Digestion** A second 10,000 - 20,000 dpm sample of each trinucleotide-enriched SVP/CAP hydrolysate was incubated, in a 1.5-ml polypropylene tube, for 1 hr at 37°C in a 1-ml reaction mix containing 50 mM Tris-HCl (pH 6.0), 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 5 mM β-mercaptoethanol, 25 units of CAP, and 6 units of nuclease P1 (Boehringer Mannheim Canada). In addition to free nucleosides, this protocol generates dinucleotides containing a cyclobutane dimer, a (6-4) photoproduct, a TA^* adduct, or two nucleosides joined by a cyclobutyl bridge. Half of each CAP/NP1 enzymatically hydrolysed sample was applied directly to a reverse-phase HPLC column. The remaining 0.5 ml was subjected to hot alkali.

c) **Hot Alkaline Treatment** A 0.5-ml fraction of each CAP/NP1 enzymatically hydrolysed sample (described above) was placed in a 1.5-ml polypropylene tube, supplemented with 55 µl of 2 N NaOH (final concentration ~0.2 N), and incubated at 90°C, for 90 min, in a circulating water bath (MgW Lauda RMT RM6, Sybron/Brinkmann). Hot alkali hydrolyses (i) the *N*-glycosyl bond of the 3'-pyrimidine of the (6-4) photoproduct, and (ii) the phosphodiester bond between the cross-linked pyrimidines of the (6-4) photoproduct (Franklin and Haseltine, 1982) but does not alter a cyclobutane dimer. The sample was then cooled, neutralized with 55 µl of 2 N HCl, and examined by reverse-phase HPLC.

E. Reverse-Phase HPLC Analysis

a) Instrumentation The HPLC instrumentation consisted of a Waters 840 control station (Waters Associates, Mississauga, ON), two Waters 510 dual piston pump, a Waters U6K universal injector, a Waters 490 Programmable Multiwavelength Detector, and Foxy fraction collector (Foxy, Lincoln, NE). The separation of [^{3}H]-labelled species was performed on a Whatman C_{18} Partisil-10 ODS-2 column (250 x 4.6 mm i.d.). All HPLC buffers were prepared using ddH_2O and filtered through Millipore type HA filters (pore size 0.45 µm; Millipore, Milford, MA).

b) Separation of [^{3}H]-labelled Species and Subsequent Analysis Each prepared 1-ml sample was injected onto the column and eluted over 40 min using a methanol gradient. The gradient employed to achieve separation of the various [^{3}H]-labelled species consisted of the following: 100% buffer A [50 mM NaH_2PO_4 (pH 4.5)] and 0% buffer B [50 mM NaH_2PO_4 (pH 4.5) containing 50% methanol (HPLC grade)] for 1 min, followed by a linear gradient from 100% to 0% buffer A and from 0% to 100% buffer B over 30 min. The 100% buffer B was maintained for 5 min, followed by a return to initial conditions in 4 min. A flow rate of 1 ml/min was maintained throughout this procedure. The first seventy 0.5-ml fractions were collected directly into plastic scintillation vials and supplemented with 5 ml of Beckman EP scintillation cocktail. The radioactivity content of each collected fraction was determined in a liquid scintillation system. The identity of several of the radioactive species under study (Thy, dT, T<>T, T<>U, d-T<p>T, and d-T<p>C) was determined based on their retention time as ascertained by others in our laboratory using the identical system (Weinfeld *et al.*, 1986; Liuzzi *et al.*, 1989). The authenticity of the nucleosides dimerized by a cyclobutyl bridge (i.e. with a cleaved intradimer phosphodiester bond; dT<>dT and dT<>dC), and of the (6-4) photoproduct-containing dinucleotides (d-T[p]T and d-T[p]C) were determined as outlined below (see Section X).

IX. Analysis of UV Adducts in Excision Fragments

A. Strong Anion Exchange HPLC Analysis Instrumentation was identical to that described above for reverse-phase HPLC. The column employed for separation was a Whatman Partisil-10 SAX column (250 x 4.5 mm i.d.) A 10,000 - 30,000 dpm sample of each TCA-soluble fraction (human and CHO only) was diluted to 1 ml with ddH_2O and injected onto the system. Separation proceeded over 80 min and employed a salt gradient. Elution conditions were as follows: 100% buffer A [0.1 mM KH_2PO_4 (pH 6.6) containing 10% ethanol (HPLC grade)] and 0% buffer B [0.4 M KH_2PO_4 (pH 6.6) containing 10% ethanol] for 5 min, followed by a linear gradient from 100 % to 0 % buffer A and from 0 % to 100 % buffer B over 30 min. The 100 % buffer B was maintained for 10 min, followed by a return to initial conditions in 10 min. A flow rate of 0.8 ml/min was maintained throughout. The first eighty 0.5-ml fractions were collected directly into scintillation vials, and their radioactivity content determined as described above. The retention times of various radioactive species under study [Thy, dT, TMP, TDP, TTP, d-TpT, d-TpTpT, $(dT)_5$, $(dT)_7$, and $(dT)_{10}$] were determined by measuring the absorbance, at 260 nm, of authentic markers (Sigma Chemical Company). In the case of dT<>dT, following verification of its structure (as outlined below), the retention time of this molecule on the SAX chromatographical system was ascertained by injecting the [^3H]-labelled species isolated from reverse-phase HPLC.

B. Basic Three-Stage Analysis Procedure Although CHO cells had large nucleotide precursor pools which contaminated the TCA-soluble material isolated from these cells, the amount of radioactivity not associated with UV lesion-containing molecules was small in comparison to the situation when [^{3}H]-labelled genomic DNA was reduced to mononucleosides plus lesion-containing trinucleotides. Therefore, for mammalian cells, the TCA-soluble fractions could be directly analyzed without first enriching for the lesion-containing oligonucleotides. However, for the TCA-soluble material isolated from human cells, subsequent analysis by the procedures outlined below required only 1,000 to 10,000 dpm per assay, whereas the equivalent analysis for material isolated from CHO cells required 10,000 to 100,000 dpm per assay. For the TCA-soluble samples derived from *E. coli*, the large amount of unincorporated thymidine was reduced by dialysis (as outlined above) prior to further study. This procedure reduced the radioactivity approximately 20-fold. However, subsequent analysis of this dialysed sample still required 100,000 to 500,000 dpm per assay.

a) Formic Acid Hydrolysis This procedure was conducted exactly as described above for trinucleotides derived from SVP/CAP digested genomic DNA.

b) SVP / CAP / NP1 Digestion Each samples of TCA-soluble material was incubated overnight at 37°C, in a 1.5-ml polypropylene tube, in a 1-ml reaction mix containing 50 mM Tris-HCl (pH 6.0), 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 5 mM β-mercaptoethanol, 0.025 units of SVP, and 25 units of CAP . Six units of nuclease P1 were added to each reaction the following morning, and incubated continued for an additional 1 hr at 37°C. Half of this enzymatically hydrolysed sample was applied directly to the reverse-phase column. The remaining 0.5 ml was subjected to hot alkaline treatment.

c) Hot Alkaline Treatment This procedure was conducted exactly as described above for the CAP/NP1 hydrolysed trinucleotides derived from enzymatically digested genomic DNA.

C. Reverse-Phase HPLC Analysis This procedure was conducted exactly as described above for the three-stage protocol utilized with genomic DNA.

D. Polyacrylamide Gel Electrophoresis

a) [^{32}P] End-labelling of Excision Fragments To aid in visualization of the lesion-containing oligonucleotides separated by polyacrylamide gel electrophoresis, the material isolated in the TCA-soluble fraction from post-UV incubated cells was first subjected to dephosphorylation, and was subsequently end-labelled with [^{32}P]. An aliquot of each TCA-soluble sample, containing ~1000 dpm, was incubated in a 50-µl reaction containing 50 mM Tris-HCl (pH 8.5), 0.1 mM EDTA, and 1 unit CAP, for 1 hr at 37°C. This reaction removed the 5'-nonradioactive phosphate(s) from all molecules. The phosphatase was then heat inactivated (75°C for 10 min), and the reaction mixture was subsequently phenol/chloroform-extracted and ethanol precipitated (as described above) to ensure the removal of all phosphatase activity. After recovery and drying of the DNA pellet, it was resuspended in a 50-µl reaction mixture consisting of 10 mM Tris acetate (pH 7.0), 10 mM magnesium acetate, 50 mM potassium acetate, 5 µCi of [γ-^{32}P] ATP (1 mCi/ml; 3,000 Ci/mmol; Amersham, Burlington Heights, IL), and 20 units of T4 polynucleotide kinase. The reaction was incubated for 1 hr at 37°C, and subsequently supplemented with 25 µl of loading dye (containing 90% formamide, 50 mM EDTA, 0.02% bromophenol blue, and 0.2% xylene cyanol).

b) Gel and Electrophoresis Conditions Thirty-three ml of a 60% acrylamide solution [containing 57 g acrylamide (ultrapure quality; International Biotechnologies, Inc., New Haven, CT) and 3 g *N,N'*-methylene bisacrylamide (ultraPURE; BRL), and made up to 100 ml with ddH$_2$O], 48 g of urea (enzyme grade; BRL), and 10 ml of 10 x TBE buffer (consisting of 0.89 M Tris base, 0.89 M boric acid, and 20 mM EDTA) were placed in a 250-ml beaker with a magnetic stir bar and mixed, with heating, to dissolve the urea. The resulting solution was filtered through Whatman No. 1 paper into a 100-ml graduated cylinder. The volume was adjusted up to 100 ml with ddH$_2$0 before transferring the mixture to a 250-ml side-arm flask. The corked flask was attached to a vacuum, and the acrylamide/urea solution was degassed for 2 min. Five hundred µl of a fresh 10% ammonium persulfate solution (made with ddH_20) and 50 µl of TEMED were added to the degassed solution with gentle mixing. The resulting mixture was poured, by means of a syringe attached to plastic tubing, between two clean siliconized vertical glass plates (20 cm x 40 cm and 20 cm x 38 cm) which were separated by 1 mm spacers on three sides. A comb (1 mm thick) was inserted into the top of the glass plates to produce wells for sample loading. After the gel had polymerized (~ 1 hr), the bottom spacer was removed from the glass plates, and the gel was placed into an electrophoresis apparatus (Dual Vertical Slab Gel Electrophoresis Cell, BioRad Laboratories, Richmond, CA) filled with TBE. After removing all air between the gel and the buffer contained in the apparatus, the gel was prerun for 10 min prior to loading of the samples. Following this prerun, approximately 25,000 cpm (as ascertained by Cherenkov counting) of each sample under study was loaded onto the polyacrylamide gel. Separation was achieved by applying 800 V for 4 hr, supplied by a Model 3000/300 Power Supply (BioRad). The glass plates were then separated, and the gel was transferred to filter paper and encased in plastic wrap. The bands were visualized after exposure to X-ray film (35 x 43 cm X-Omat; Kodak Scientific Imaging Film; Kodak Canada Inc., Toronto, ON) in a Kodak X-Omat Cassette. Exposure time varied from 30 min to overnight depending on the efficiency of incorporation of the radioactivity into the oligonucleotides. The lengths of the oligonucleotides in the samples were determined by running, in a parallel lanes, a series of [^{32}P] end-labelled markers of known length. These markers were kindly supplied by Dr. Michel Liuzzi.

X. Verification of the Identity of Dimerized Dinucleotide(side)s

A. T<>U, T<>T, dT<p>C, dT<p>T The identity of these radioactive species was determined based on their retention time as ascertained by others in our laboratory using the identical system (Weinfeld *et al.*, 1986; Liuzzi *et al.*, 1989).

B. dT[p]Py Under the reverse-phase HPLC conditions employed, the radioactivity eluting at fractions 29-34, when enzymatically digested, UV-irradiated and unrepaired genomic DNA was assessed, contained (6-4) photoproducts. The authenticity of this material was ascertained by hot alkaline treatment.

A sample (5 x 10^7 dpm) of UV-irradiated and unrepaired human high-molecular-weight DNA was subjected to the primary digestion and dialysis procedure outlined above. Approximately 2.5 x 10^5 dpm remained following dialysis. This material was hydrolysed with NP1/CAP, and subsequently applied to the reverse-phase HPLC column. Following collection of seventy 0.5-ml effluent samples, fractions 29-34, which contained ~8 x 10^3 dpm, were pooled and taken to dryness in a SpeedVac concentrator. The dried pellet was resuspended in 1 ml of ddH_20. One-third of the resulting suspension was reinjected onto the HPLC to confirm that the isolation procedure had not altered the retention time of the species in question. A second portion was subjected to formic acid hydrolysis, as outlined above, prior to analysis by reverse-phase HPLC. The remaining one-third was treated with hot alkali in a manner identical to that indicated above for genomic DNA.

Reinjection of the untreated sample confirmed that the isolation procedure had not alter the retention time of the species in question. Formic acid hydrolysis verified that this material did not contain cyclobutane dimers. However, hot alkaline treatment indicated that (6-4) photoproducts were present. As outlined above, hot alkali hydrolyses (i) the *N*-glycosyl bond of the 3'-pyrimidine of a (6-4) photoproduct, and (ii) the phosphodiester bond between the cross-linked pyrimidines of a (6-4) photoproduct (Franklin and Haseltine, 1982). This would result in a less hydrophobic compound. As this treatment moved 100% of the isolated material to the beginning of the HPLC chromatogram, it was surmised to consist of only (6-4) photoproducts-containing dinucleotides. This peak was later shown by Dr. Michel Liuzzi (Liuzzi *et al.*, submitted for publication) to display other characteristics peculiar to (6-4) photoproducts (Lippke *et al.*, 1981; Taylor *et al.*, 1988). This material exhibited fluorescence, with a maximum emission at 408-nm, (measured with a Perkin-Elmer LS5 fluorescence spectrophotometer) and was prone to photolysis (upon irradiation with 45 kJ/m^2 of 366-nm UV light).

C. dT<>dC, dT<>dT The radioactive species, dT<>dC and dT<>dT, were found to migrate at fractions 28-31 and 35-40, respectively, under the reverse-phase chromatographical condition employed. The identity of these species was identified by photochemical reversal.

A sample of TCA-soluble material (~10^5 dpm) derived from post-UV incubated (24 hr) human cells was subjected to SVP/CAP/NP1 hydrolysis, and the resultant species were separated by reverse-phase HPLC, as outlined above. The species eluting at fractions 28-34 and 35-40 were reserved, pooled into the two respective samples, and taken to dryness in a SpeedVac concentrator. Each dried pellet was resuspended in 1 ml of ddH_20. Because the material in the first of these two peaks was known to contain (6-4) photoproducts, it was subjected to hot alkaline treatment, as outlined above, and subsequently reinjected onto the reverse-phase HPLC. As (6-4) photoproducts undergo modification in the presence of hot alkali, and as a result of this treatment migrate at the beginning of the chromatogram, the material still migrating at fractions 28-31 following such treatment was presumed to be free of this species. These fractions were pooled, dried, and resuspended in ddH_2O, as indicated above.

The (6-4) photoproduct-free samples (i.e. fractions 28-31 recollected following hot alkaline treatment, as well as fractions 35-40 from the original HPLC run) were each divided into three aliquots. One-third of each sample was reinjected onto the HPLC to again ensure that the isolation procedures had not altered the respective retention times. A second aliquot was subjected to formic acid hydrolysis as outlined above. The remaining one-third of each sample was exposed to a photoreversing fluence of 254-nm light (Haseltine *et al.*, 1980) prior to reinjection onto the HPLC. For this purpose, each 330-µl sample was placed in a 40-mm tissue culture dish (Lux Scientific Corp.) and supplemented with 570 µl of ddH_2O and 100 µl of ethanol. The contents were then exposed, with an open lid, to 5.5 kJ/m^2 of 254-nm light, using the same UV source as described above for the irradiation of cells. Each sample was then transferred to a 1.5-ml polypropylene tube, taken to dryness under vacuum in a SpeedVac Concentrator, and resuspended in 1 ml of ddH_2O before injecting onto the reverse-phase HPLC.

Reinjection of the untreated samples again confirmed that the isolation procedures had not alter the retention time of the species in question. Formic acid hydrolysis verified that both peaks contained cyclobutane dimers. Fractions 28-31 were established to consist of T^C cyclobutane dimers-containing dinucleotides. Fractions 35-40 constituted T^T-containing dinucleotides. Photochemical reversal caused a shift in the retention time of both species to that of thymidine. (As the DNA was labelled only on thymidine, this would be the only nucleoside detected.) Since monomerization of the cyclobutyl bridge in both instances produced a mononucleoside, and since the species were known to involve T^C and T^T cyclobutane dimers, respectively, the compounds in question were surmised to constitute the modified dimer-containing species, dT<>dC and dT<>dT.

D. **TA* Photoproduct** A radioactivity-labelled marker for identifying the retention time of the TA*-containing dinucleotide on the reverse-phase chromatogram was isolated by the following procedure. The alternating copolymer, d(AT)$_n$, was purchased from Sigma Chemical Company (average length of this copolymer was estimated to be 500 nt). One µg of this polymer was diluted into a 100-µl reaction mixture containing 0.1 M HEPES (pH 6.9; Sigma Chemical Company), 10 mM $MgCl_2$, 2.5 mM dithiothreitol (Sigma Chemical Company), 70 mM KCl, 50 µM dTTP, 1 mM dATP, and 5 µl [^{3}H]-TTP (1Ci/ml; 41 Ci/mmol; NEN Canada, Montreal, PQ). After supplementing with 20 units of DNA polymerase I (Promega, Madison, WI), the reaction was incubated at 20°C, in a Circulating Cooling Water Bath (MgW Lauda RMT RM6, Sybron/Brinkmann), for 20 hr. As no exogenous primer was present to initiate second-strand synthesis, the incubation conditions employed presumably allowed hairpin loop formation at the 3' terminus to begin synthesis of the radioactively-labelled second strand. The final double-stranded radioactively-labelled substrate had a specific activity of 170 dpm/pmol thymine and a concentration of 214 µM nucleotides).

Two hundred µl of the double-stranded [^{3}H]-labelled d(AT)$_n$ was mixed with 800 µl of ddH_20 in a 40-mm Petri dish (Lux Scientific Corp.), and irradiated, with an open lid, with a total fluence of ~10 kJ/m^2 of 254-nm light. (The UV light source was identical to that used for irradiation of cells as outlined above.) Following irradiation, the contents were transferred to a 1.5-ml polypropylene tube, and taken to dryness, under vacuum, in a SpeedVac Concentrator. After resuspending in 855 µl of ddH_2O, the contents were supplemented with 100 µl of 10x concentrated hydrolysis buffer [containing 200 mM Tris-HCl (pH 7.5), 40 mM $MgCl_2$, and 20 mM $CaCl_2$], 15 µl of 10 units/µl staphylococcal nuclease, 20 µl of 10 units/µl DNase I, 10 µl of 0.0025 units/µl SVP, and 2 µl of 10 units/µl CAP, and incubated overnight at 37°C (in a Lauda M20 Circulating Water Bath). The resulting reaction mixture was dialysed overnight, using 3 kDa MW cut-off prepared dialysis membrane, against 2 *l* of ddH_2O, with H_2O changes every 6 hr. One-half (~500 μl) of the material retained in the dialysis bag was directly analyzed by reverse-phase HPLC (using the same gradient conditions described above). The remaining 500 μl were subjected to hydrolysis by NP1/CAP for 1 hr at 37°C in a 1-ml reaction mix containing 50 mM Tris-HCl (pH 6.0), 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 5 mM β-mercaptoethanol, 25 units of CAP, and 6 units of NP1, and subsequently analyzed by reverse-phase HPLC.

As thymine and adenine are the only contiguous bases, presumably dimerization of adjacent nucleotides would generate only TA and AT lesions. However, according to others (Bose *et al.*, 1983; Bose *et al.*, 1984; Bose and Davies, 1984; Kumar and Davies, 1987), the major photoproduct induced in $d(AT)_n$, using the same experimental conditions as employed here, is the TA^* photoproduct. Therefore, it was surmised that the novel species migrating at fraction 58, upon analysis of the staphylococcal nuclease/DNase I/SVP/CAP hydrolysed UV-irradiated $d(AT)_n$, was the trinucleotide, d-ApT{p}A. In further support of this deduction, subsequent NP1/CAP hydrolysis of this material resulted in a shift in retention time of the novel species, but not a reduction in the percentage of total radioactivity associated with it. As NP1 removes the 5'-nucleotide from a 3'-lesion-containing trinucleotide, this 5-nucleotide must have been unlabelled and was therefore adenosine. The lesion-containing dinucleotide, d-T{p}A, generated by NP1/CAP hydrolysis, eluted at fraction 48.

XI. Calculation of Absolute Number of UV Photoproducts Induced in and Excised from Cellular DNA

A. Removal from High-Molecular-Weight DNA The following assumptions were made in the calculation of the absolute number of thymidine-containing UV lesions present in human genomic DNA: (i) in an asynchronous population, each human diploid fibroblast has 6×10^{-12} g (i.e. 3.6×10^{12} daltons) of genomic DNA (Sober, 1968); (ii) assuming the average molecular weight of a mononucleotide to be 327 daltons, there are therefore 1.10×10^{10} nucleotides per genome; (iii) in human DNA, the fraction of nucleotides containing thymine is 0.29 (Sober, 1968); hence the number of thymine residues per genome equals 3.19×10^9; (iv) for calculation involving T^T dimers, the percentage of radiolabel is divided by 2 since either Thy can be labelled; (v) the vast majority of thymidine-containing (6-4) photoproducts are T[p]C (Lippke *et al.*, 1981; Brash and

Haseltine, 1982; Franklin *et al.*, 1982) and therefore no correction has been made for T[p]T.

In making calculations for CHO genomic DNA, it was assumed that the amount of DNA per cell, and the fraction of nucleotides containing thymine, were approximately the same as for human cells. The analogous calculations for *E. coli* were based on a genome size of 4.2 x 10^6 bp and a thymine content of 0.25 (Lewin, 1985); therefore the number of thymine residues per *E. coli* genome equals 2.1 x 10^6.

To calculate the absolute numbers of the various UV photoproducts found in genomic DNA (d-T<p>C, d-T<p>T, dT<>dC, dT<>dT, d-T[p]Py, and d-T{p}A), the percentage of counts retained after dialysis of a particular DNA sample, was multiplied by the percentage retained by DEAE-Cellulose (where applicable), and by the percentage of radioactivity representing each particular species in the enriched sample as ascertained by reverse-phase HPLC chromatography. This calculation determined the percentage of total radioactivity represented by each photoproduct in the DNA sample in question, and was subsequently multiplied by the number of thymine residues per genome, in order to compute the absolute number of each type of lesion present in this genomic DNA sample.

Since the DNA in these experiments was labelled in thymidine, the frequency of cytosine-cytosine lesions could only be estimated from the work of others. Calculation involving C^C cyclobutane dimers were based on the assumption that these dimers occur with a frequency of 0.24 x T^C frequency (Weinfeld *et al.*, 1986). To estimate the number of C[p]C photoproducts, their frequency relative to T[p]C and T[p]T was extrapolated from data accumulated on the frequency of the various UV-lesions occurring at specific sequences in irradiated *E. coli* (Brash and Haseltine, 1982) or human cells (Brash *et al.*, 1987). These studies imply that C[p]C (6-4) photoproducts occur with a frequency of 0.27 x (T[p]C + T[p]T).

B. Accumulation in Excision Fragments To calculate the absolute number of UV lesions occurring in excision fragments, the same assumptions, as indicated above for genomic DNA, were employed. Calculations, therefore, involved multiplying the percentage of total radioactivity represented by the TCA-soluble material, by the percentage of this TCA-soluble radioactivity associated with each UV adduct. This gave a percentage of the total radioactivity represented by each type of UV lesion, and was subsequently multiplied by the number of thymine residues in the genome to give an absolute number of excised photolesions.

APPENDIX B - DETAILED METHODOLOGIES UTILIZED IN THE DEVELOPMENT OF AND *IN VITRO / IN VIVO* SYSTEM TO ASCERTAIN THE CELLULAR FUNCTION OF INTRADIMER PHOSPHODIESTERASE

I. Substrate Construction

A. Vector Sequence

a) Amplification and Isolation of Plasmid DNA One ng of the M13-derived vector, pGEM-7Zf(-), was purchased from Promega (Madison, WI). DH5α competent *E. coli* cells (BRL Inc., Burlington, ON) were transformed with this plasmid in the following manner. One hundred µl of competent cells, previously frozen and stored at -20°C in 100 µl aliquots in 400-µl polyethylene tubes (Eppendorf, Brinkmann), were thawed on ice. These cells were supplemented with 5 µl (0.05 ng) of plasmid DNA, gently mixed, and incubation continued, on ice, for 45 min. Following heat-shock treatment of this cell/plasmid mixture by placement in a 42°C circulating water bath for 45 seconds, it was added to 1 ml of Luria broth (LB) [consisting of 10 g/*l* bacto-tryptone (Difco Laboratories), 5 g/*l* bacto-yeast extract (Difco Laboratories), and 10 g/*l* NaCl, prepared with ddH$_2$O and sterilized by autoclaving], in a 16 x 125 mm disposable borosilicate glass culture tube (Fischer Scientific), and incubated for 1 hr at 37°C, without shaking. Three dilutions (1/10, 1/50, 1/100) were then plated on each of three LB + ampicillin agar plates [consisting of 10 g/*l* bacto-tryptone, 5 g/*l* bacto-yeast extract, 10 g/*l* NaCl, 15 g/*l* of bacto-agar (Difco Laboratories) and 100 µg/ml ampicillin, prepared with ddH$_2$O, and sterilized by autoclaving; each 150 x 15-mm plate contained 30-35 ml of media]. After overnight incubation at 37°C, an Amp^R colony was selected and used to inoculate 10 ml of liquid LB in a 20 x 150-mm culture tube. This was incubated overnight, with vigorous shaking, at 37°C.

The following morning, 1 ml of the overnight culture was added to 1 *l* of LB and incubated in a 2-*l* flask, with aeration, at 37°C. After the culture had reached an OD$_{600}$ of 0.6, cells were harvested by centrifugation at 3500*g* for

7 min (Nalgene 250-ml bottles, Nalge Company; GSA rotor; Sorvall RC-5C Refrigerated SuperSpeed Centrifuge) and resuspended in 30 ml of PebI [50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0)] containing 10 mg/ml lysozyme. After incubating on ice for 10 min, the cell suspension was supplemented with 70 ml of PebII (0.2 N NaOH, 1% SDS), and incubation continued for an additional 10 min. Fifty ml of 3 M KAc (pH 4.8) was added to the cell lysate, which was subsequently left on ice for 15 min. Bacterial DNA and proteins were pelleted by centrifugation at 10,000*g* for 15 min (GSA rotor, Sorvall RC-5C Refrigerated SuperSpeed Centrifuge). The supernatant was filtered through hospital gauze (4-ply, 10 cm x 10 cm; Johnson and Johnson, Vancouver, B.C.) to remove unpelleted debris. Plasmid DNA was precipitated from the resulting clear supernatant with 2 volumes of ice-cold 100% ethanol. After recovery by centrifuging at 10,000*g* for 10 min, the pellet was resuspended in 4 ml TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA]. This suspension was then added to 8.2 ml of a saturated CsCl (ultraPURE, BRL) solution (in TE) and supplemented with 400 µl of an ethidium bromide solution (10 mg/ml), and finally sealed inside a Sorvall 11.5 ml crimp-top tube. Gradient formation was achieved by spinning at 55,000 rpm (Sorvall RC70 Centrifuge) in a fixed angle rotor (Sorvall T865) at 20°C for 48 hr. The lower band (CCC DNA) was recovered by entering into the side of the tube with a 22.5-gauge needle (Becton Dickinson) attached to a 3 cc syringe (Becton Dickinson). The ethidium bromide was removed by extracting 5 times with water-saturated n-butanol. The top (organic) phase was withdrawn and discarded with each successive extraction. The CsCl was removed by dialysis against 2 *l* of TE overnight. The final concentration of the purified plasmid was 7.7 mg/ml.

Isolation of the desired vector fragment required double-digestion with two restriction enzyme, *Nsi*I and *Sph*I, each of which required a different reaction buffer. For simplicity, the vector was restricted in a single step (with both enzymes present), in the buffer recommended for use with *Nsi*I. However, prior to performing this reaction, the ability of increasing amounts of each enzyme to completely digest 10 µg of vector sequence, in the buffer supplied with *Nsi*I, was first assessed. Furthermore, as restricting in non-ideal conditions can result in products which cannot re-ligate, this ability was also evaluated for each restricted sample.

Ten µl of a 1 µg/ml dilution of the pGEM vector was transferred to a 400-µl polyethylene tube containing 69 µl of ddH_2O. This DNA solution was then supplemented with 10 µl of 10 x *Nsi*I buffer [supplied with the restriction enzyme and containing 100 mM Tris-HCl (pH 7.9), 1.5 M NaCl, 100 mM $MgCl_2$, and 60 mM β-mercaptoethanol], 10 µl of a 1 mg/ml BSA preparation (BRL), and 0.1, 0.5, 1, or 5 units of *Nsi*I (Promega; supplied at a concentration of 20 units/µl; to add less than 20 units to a reaction mixture, the enzyme was diluted in ddH_2O just prior to usage). This series of reaction was also repeated using, instead of *Nsi*I, 0.1, 0.5, 1, or 5 units of *Sph*I (Promega; supplied as a 15 units/µl solution). Digested samples were inspected by gel electrophoresis (minigel Model H6 Horizontal System, BRL; Model 100 Power Supply, BRL) on a 0.8% agarose (ultraPURE, BRL) gel submerged in 1 x TAE buffer (consisting of 40 mM Tris-acetate and 1 mM EDTA). Prior to loading onto the gel, a 10-µl aliquot of each reaction was supplemented with 5 µl of loading dye [containing 15% Ficoll (Type 400; Pharmacia), 0.25% bromophenol blue, and 0.25% xylene cyanol FF]. In a parallel lane, a 10-µl sample, containing 1 µl of undigested pGEM DNA, 4 µl of loading dye, and 5 µl of ddH_2O, was run as a standard. Separation was effected by applying 50 V for 1 hr. The gel was then transferred to 30 ml of a solution containing 0.5 µg/ml ethidium bromide, and left for 30 min. The stained DNA was visualized using a UV Transilluminator, UVP incorp. (San Gabriel, CA) and recorded on polaroid film. These preliminary digestion reactions confirmed that *Sph*I could completely restrict the pGEM vector in *Nsi*I buffer at a ratio of 1 unit of enzyme to 10 µg of plasmid DNA. The ability of this restricted DNA to re-ligate was then assessed.

Twenty µl of the *Nsi*I or *Sph*I restricted pGEM vector (containing ~2 µg of DNA) was supplemented with 2.5 µl of 10 x ligase buffer [containing 0.5 M Tris-HCl (pH 7.5), 0.1 M $MgCl_2$, 0.1 M dithiothreitol, and 0.16 M ATP] and 2.5 µl of 0.1 Weiss unit/µl T4 DNA ligase (Promega). This reaction mix was incubated overnight at 16°C (MgW Lauda RMT RM6 Circulating Cooling Water Bath). The following morning, 10 µl of each reaction was supplemented with

5 μl of loading dye, and analyzed by agarose gel electrophoresis in the presence of ethidium bromide (0.5 μg/ml), as otherwise indicated above. Ethidium bromide was incorporated into the gel in this instance since re-ligation of the restricted vector would generate a covalently-closed circular molecule which was not supercoiled. In the absence of ethidium bromide, this species would migrate very close to the linear molecule, at the position of open-circle DNA. This would make it impossible to distinguish between those molecules which had undergone re-ligation of one phosphodiester bond, and those which had undergone re-ligation of both. However, ethidium bromide induces positive-supercoiling into a CCC DNA. Therefore, if both phosphodiester bonds had reformed, this species would migrate to the same position as the native negatively-supercoiled plasmid.

A 1-μl sample of unrestricted pGEM DNA was run in a parallel lane and served as a reference molecule. This experimented indicated that the pGEM plasmid DNA, restricted with *Sph*I in a buffer system designed for use with *Nsi*I, had normal capacity to re-ligate. Therefore, the pGEM vector to be used for the construction of the substrate molecule, was double-digested with both enzymes using this pretested buffer system. Both enzymes were present in this reaction at nearly 3-fold the enzyme:DNA ratio which had been shown in preliminary experiments to completely digest the vector DNA.

Five hundred and sixty μg of pGEM vector was double-digested with *Nsi*I and *Sph*I in a 1-ml reaction containing 10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 10 mM $MgCl_2$, 0.1 mg/ml BSA, 200 units *Nsi*I, and 200 units *Sph*I, overnight at 37°C. Digested samples were inspected in the morning by agarose gel electrophoresis as outlined previously. This reaction produces two vector fragments, of 83- and 2917-bp fragments. Since only the larger vector sequence was required for subsequent steps, and because it was important to ensure that the starting molecule could not be regenerated, these two fragments were separated by gel filtration chromatography.

A 30-ml column (15 x 2 cm; Pharmacia) was packed with sepharose CL-6B (Pharmacia) and subsequently equilibrated with 0.1 M Tris-HCl (pH 7.5), and 10 mM EDTA. The flow rate was set at 0.1 ml/min, using a peristaltic pump (Pharmacia), and was maintained both during equilibration and after loading of the digested vector DNA. After reserving 5 µl of the 1-ml restricted DNA sample, the remainder was applied to the column, and 5-min (~0.5 ml) fractions of the effluent were collected (Pharmacia fraction collector). The amount of DNA in each fraction was then estimated by measuring the OD_{260} of the entire fraction. Those comprising the first UV_{260} peak were pooled and dialysed (3 kDa MW cut-off tubing) overnight against a large volume of ddH_20. Subsequent agarose gel electrophoresis confirmed that this material comprised the longer 2917-bp fragment. The final concentration of this vector fragment was 104 µg/ml.

To ensure that the 83 bp-fragment had been removed from the gel filtration-purified 2917-bp vector sequence, re-ligation of this material was tested. At the same time, the 5-µl reserved sample of unfractionated *Nsi*I/*Sph*I restricted pGEM was also subjected to the same ligation conditions. This latter reaction served not only as a control for the ligation reaction itself, but also functioned as a second test of the quality of the termini produced by *Sph*I digestion in a *Nsi*I buffer system. Each reaction contained 3 µl of 10 x ligase buffer (described above), 2.8 µg of DNA (5 µl of the unfractionated restricted vector or 27 µl of the sample collected from the sepharose column), 1 µl of 0.1 Weiss units/ml T4 DNA ligase, and ddH_2O (if necessary) to bring the volume up to 30 µl. Incubation was carried out, overnight, at 16°C, and reaction products were assessed by agarose gel electrophoresis, in the presence of ethidium bromide, the following morning. This experiment clearly indicated that, whereas the unfractionated material was capable of reforming CCC DNA, the fractionated DNA was not.

B. Oligonucleotides for Ligation to the 2917-bp pGEM Vector Fragment The five oligonucleotides used in the construction of substrate molecules were purchased from the DNA Synthesis Facility in the Department of Microbiology, University of Alberta. These were designated AGA-4, AGA-6, AGA-7, AGA-8, and AGA-9. AGA-4 is a 5'-phosphorylated 12-mer of sequence 5'-p-GTCCGGAGTGCA. AGA-6 is a non-phosphorylated 11-mer with the sequence 5'-GCAAGTTGGAG. AGA-7 is a 5'-phosphorylated 23-mer used as the strand complementary to AGA-4 and AGA-6. It has the sequence 5'-p-CTCCGGACCTCCAACTTCCACTG. AGA-8 and AGA-9 together comprise AGA-6. AGA-8 is a 5'-phosphorylated 5-mer of sequence 5'-p-TGGAG.

AGA-9 does not have a terminal phosphate and has the sequence 5'-GCAAGT. Approximately 1 µmol of each oligonucleotide was synthesized, and all were received in a dried state. Upon arrival, all were dissolved in 1 ml of ddH_2O. The concentration of was each determined by diluting a 5-µl aliquot with 995 µl ddH_2O and measuring the OD_{260}. The concentrations of these oligomers varied between 0.7 and 3.4 mg/ml.

a) Purification of AGA-4 and AGA-7 Prior to usage, AGA-4 and AGA-7 were purified on a NAP-5 column (Pharmacia). These columns are prepacked and disposable, and contain the gel filtration matrix, Sephadex G-25. Small molecules, such as salts, are left on the column if only a 1.5-ml volume is allowed to pass through the column matrix. The column was first equilibrated with 3 volumes of TE buffer [10 mM Tris-HCl (pH 7.0), 1 mM EDTA], after which 0.5 ml of an oligonucleotide solution was applied, and the first 0.5 ml of effluent was discarded. The purified oligomer was then eluted with 1 ml of TE and stored at 4°C for further use. Because manipulations of AGA-6, AGA-8, and AGA-9 involved HPLC purification steps, these oligomers were not cleaned by this method.

b) Irradiation of AGA-6 and Isolation of Cyclobutane Dimer-Containing Molecules The AGA-6 oligomer contains only one dipyrimidine site, adjoining TT residues 6 and 7 nucleotides from the 5'-terminus. This is, therefore, the only position where a cyclobutane dimer can be induced.

AGA-6 was irradiated with 10 kJ/m^2 of 254-nm light in the following manner. Approximately 30 nmol of AGA-6 was diluted to a total volume of 20 ml with ddH_2O, and transferred to a 150-mm tissue culture dish. This dish was then placed, with an open lid, under a germicidal lamp which emits 97% of its radiant energy at the 254-nm wavelength (see Appendix A for lamp specifications). The solution was stirred continuously to ensure uniform irradiation. The contents were then transferred to a 30-ml round-bottom flask, taken to dryness in a rotoevaporator (Rotavapor REIII; Büchi, Switzerland), and resuspended in 50 µl of ddH_2O. This procedure was repeated 10 times in all.

Because of the kinetics of cyclobutane dimer formation, only a minority of the AGA-6 oligonucleotides irradiated with 254-nm light will contain a cyclobutane dimer. [In poly(dT), a dose of 10 kJ/m^2 of 254-nm light dimerizes approximately 35% of the thymine. In native human DNA, the maximum yield of T^T dimers which can be achieved with 254-nm light is approximately 7% (Rahn and L.C. Landry, 1971).] To obtain a pure population of cyclobutane dimer-containing oligomers, this irradiated material was subjected to purification by HPLC chromatography. The protocol employed entailed a slight modification of the procedure previously described by Banerjee *et al.* (1988).

Separation of the T^T-containing oligonucleotides from the parent AGA-6 and from other photoproduct-containing molecules [such as a small number of (6-4) photoproduct-containing oligomers] was achieved using the same HPLC instrumentation described previously, with the exception of the reverse-phase column used. Instead of an ODS-2 reverse-phase column, the column specifically employed for this purpose was an end-capped Nova-Pak C_{18} reverse-phase column (250 x 4.6 mm i.d.; Waters, Mississauga, ON). The column was first equilibrated with 7.5% acetonitrile in 0.1 M triethylammonium acetate. Each prepared 1-ml sample was then injected onto the column and eluted over 40 min using an acetonitrile (HPLC grade) gradient. The gradient employed to achieve separation of the lesion-containing oligomers and the parent molecule consisted of the following: a linear gradient from 90% buffer A (0.1 M triethylammonium acetate) and 10% buffer B (0.1 M triethylammonium acetate, 75% acetonitrile) to 88% buffer A and 12% buffer B over 20 min, followed by a second linear gradient from 88% to 0% buffer A and from 12% to 100% buffer B over 10 min, and followed by a third linear gradient back to initial conditions. A flow rate of 1 ml/min was maintained throughout this procedure, and the retention time of the various separating species was monitored by the UV absorbance (OD_{260}) of the effluent. Eighty 0.5-ml fractions were collected directly into 1.5-ml polypropylene tubes. The fractions corresponding to the presumed parent molecule were pooled, as were those suspected of containing the oligomer with a T^T dimer. Both pools were subsequently taken to dryness in a rotoevaporator, which also served to remove the acetonitrile. Each pooled sample was resuspended in 500 µl of ddH_2O and dialysed against 2 *l* of ddH_2O overnight, with water changes at 4 and 8 hr, to reduce the high concentration of triethylammonium acetate.

Those two samples containing either parent molecule or cyclobutane dimer-containing oligomers were identified as follows. In the former case, the purchased material served as a marker for those oligonucleotides unaltered by the UV treatment. For the dimer-containing molecules, their identity was confirmed by both photochemical and photoenzymatic reversal. For the former procedure, a 1-µg (~300 pmol) aliquot of the sample suspected of comprising the purified T^T-containing 11-mer, was diluted to a final volume of 1 ml with ddH_2O. This was then placed in a 40-mm tissue culture dish and exposed, with an open lid, to a total fluence of 5.5 kJ/m^2 of 254-nm light. The irradiated material was then reinjected directly onto the Nova-Pak HPLC column, and analyzed by the same gradient protocol as described above. Regeneration of the parent peak confirmed the identity of this material as cyclobutane dimer containing oligomers.

Confirmation of the above result was obtained by observing the effect of photoenzymatic reversal on the isolated material. For this procedure, 1 µg (~300 pmol) of the purified oligomer was diluted into a 200-µl reaction mixture containing 50 mM Tris (pH 7.5), 125 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol and 10 µg of photolyase (purified in our laboratory by John Chan; the cloned gene was supplied by Dr. Aziz Sancar). This mixture was placed under fluorescent light for 1 hr, and subsequently analyzed by HPLC reverse-phase as indicated above. This reaction, too, regenerated the parent molecule.

Once this species had been definitely identified, the HPLC purification procedure was repeated two additional times to ensure that all contaminating molecules had been removed. The parent molecule, AGA-6, was also subject to these extra purifications to ensure that each of these two species had been treated in equivalent manners.

c) 5'-Phosphorylation of AGA-6 and its Cyclobutane Dimer-Containing Derivative For the eventual ligation of the various oligonucleotides to the pGEM vector sequence, each must carry a 5'-phosphate. Therefore, as AGA-6 was purchased without this modification, following purification of both AGA-6 and T^T-containing AGA-6 by three successive rounds of HPLC chromatography, they were subjected to 5' phosphorylation. This was executed in two ways: by kinasing with (i) unlabelled ATP, to generate a population of molecules for eventual ligation to the pGEM vector sequence; or (ii) [γ-^{32}P] ATP, to generate a labelled marker to aid in HPLC isolation of the phosphorylated derivatives and to assist in future experiments (such as modification by intradimer phosphodiesterase), by enabling very small quantities of these species to be used at any one time.

Nonradioactive phosphorylation of both AGA-6 and its dimer-containing derivative was accomplished in the following manner. Two nmol of oligomer were kinased in a 1-ml reaction mixture consisting of 10 mM Tris acetate (pH 7.0), 10 mM magnesium acetate, 50 mM potassium acetate, 50 nmol unlabelled ATP, and 50 units of T4 polynucleotide kinase. The reaction mixture was incubated overnight at 37°C, and was repeated 10 times in total for each oligomer. For radioactive end-labelling, 50 pmol of oligomer were kinased in a 50-µl volume containing the same components as indicated above, except that instead of unlabelled ATP, 5 µCi of [γ-^{32}P] ATP (1 mCi/ml; 3,000 Ci/mmol; Amersham) was present. This reaction was incubated at 37°C for 1 hr.

As the presence of non-phosphorylated molecules would interfere with the efficiency of ligation, these molecules were isolated from their 5'-phosphorylated derivatives by reverse-phase HPLC chromatography. Each 1-ml sample of non-radioactively kinased oligonucleotide was injected onto the same Nova-Pak reverse phase C_{18} column as described previously, and eluted over 40 min using an acetonitrile gradient. This gradient scheme varied slightly from the one described earlier. The initial conditions of 92% buffer A (0.1 M triethylammonium acetate) and 8% buffer B (7.5% acetonitrile in 0.1 M triethylammonium acetate) were maintained for 2 min. This was followed by a linear gradient from 92% to 88% buffer A and from 8% to 12% buffer B over 18 min, followed by second a linear gradient from 88% to 6% buffer A and from 12% to 94% buffer B over the subsequent 10 min, and finally a return to initial conditions over 10 min. A flow rate of 1 ml/min was maintained throughout and eighty 0.5-ml fractions were collected. The fractions containing the phosphorylated molecule were identified by the retention time of the [^{32}P]-labelled counterpart. These were each pooled, taken to dryness in a rotoevaporator, resuspended in ddH_2O, and finally dialysed overnight against water, all as described previously. The final concentrations of 5'p-AGA-6 and T^T-containing 5'p-AGA-6 were 10 pmol/µl and 2 pmol/µl, respectively. Approximately 4 nmol of each was isolated. Radioactively end-labelled oligomers were not subject to this purification step.

d) Isolation of Oligonucleotides with a Modified Cyclobutane Dimer

Initial attempts to generate an AGA-6 derivative containing a modified dimer entailed treatment of the HPLC-purified oligomers with human intradimer phosphodiesterase (IDP). This activity had been previously isolated by Dr. Michel Liuzzi and John Chan of our laboratory from human liver. Radioactively end-labelled AGA-6 and T^T-containing AGA-6 were employed for these experiments. One pmol (~8 x 10^5 cpm) of an oligonucleotide was incubated in a 50-µl reaction mix containing 25 mM NaAc (pH 5.5), 2.5 mM $MgCl_2$, 1 mM β-mercaptoethanol, and 0.5 µg of human IDP at 37°C for 1 hr. After heating to 100°C for 10 min, an aliquot (~8 x 10^4 cpm) of the reaction mixture was analyzed by HPLC using the same gradient system as employed for the isolation of phosphorylated AGA-6 derivatives. As this procedure revealed no evidence of alteration in the structure of either IDP-treated oligonucleotide, the reaction mixtures were analyzed by polyacrylamide gel electrophoresis following: (i) no further treatment; (ii) photoenzymatic reversal; and (iii) photochemical reversal. In conjunction with experiments involving photoreversal of IDP-treated AGA-6 and T^T-containing AGA-6, samples which had not been previously exposed to IDP were subjected to the same procedures and served as controls. In addition, a set of experiments were performed in which photolyase was present with the oligonucleotide at the time of the original IDP treatment. The reasoning for this latter endeavour was that, as pre-binding of photolyase to a cyclobutane dimer stimulates repair of these lesions by the UvrABC complex (Sancar *et al.*, 1984; Sancar and Smith, 1989), it may also stimulate hydrolysis of the intradimer phosphodiester bond by IDP.

For treatment of the oligonucleotides with IDP in the presence of photolyase, 1 pmol (~8 x 10^5 cpm) of [^{32}P] end-labelled oligonucleotide was incubated in a 10-µl reaction mix containing 10 mM Tris (pH 7.5), 125 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol and 2 µg of photolyase for 30 min, at room temperature, in the dark. This reaction was then supplemented with 5 µl of 10 x human IDP buffer [containing 250 mM NaAc (pH 5.5), 25 mM $MgCl_2$, and 10 mM β-mercaptoethanol], 2 µl of 1 M NaCl, 32 µl ddH_2O, and 0.5 µg human IDP, and incubated, in the dark, at 37°C for 1 hr. After heating to 100°C for 10 min, a 5-µl sample was set aside for PAGE analysis. Two additional aliquots were subjected to photochemical and photoenzymatic reversal.

For all samples subjected to photoenzymatic reversal (AGA-6, T^T-containing AGA-6, IDP-treated AGA-6, IDP-treated T^T-containing AGA-6, photolyase-prebound, IDP-treated AGA-6, and photolyase-prebound IDP-treated T^T-containing AGA-6) ~8 x 10^4 cpm (corresponding to ~100 fmol or 300 pg) of oligonucleotide were incubated in a 100-µl reaction mix, containing 50 mM Tris (pH 7.5), 125 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 2 µg of photolyase under fluorescent lighting for 1 hr at room temperature. This mixture was then dot dialysed (see previously) to decrease the NaCl content before reducing the reaction volume to ~5 µl in a SpeedVac Concentrator. This reduced sample was supplemented with 5 µl of loading dye and set aside for PAGE analysis.

Photochemical reversal of oligonucleotides was performed by diluting a 8 x 10^4 cpm sample with ddH_2O up to a total volume of 1 ml. As done previously, this was transferred to a 40-mm tissue culture dish, irradiated with 5.5 kJ/m^2 of 254-nm light, and the volume reduced in a SpeedVac Concentrator before finally resuspending in 5 µl of ddH_2O plus 5 µl of loading dye. A 8 x 10^4 cpm aliquot of each of the six samples listed above, not subjected to photoreversal, were likewise suspended in a final volume of 5 µl with ddH_2O and supplemented with 5 µl of loading dye.

Polyacrylamide gel analysis was employed using the same conditions and equipment as described earlier [20% acrylamide denaturing (8 M urea), 20 cm x

40 cm gel; BioRad Dual Vertical Slab Gel Electrophoresis]. The eighteen 10-µl prepared samples described above, were loaded onto two gels and electrophoresed for 4 hr at 800 V, as described previously. The gels were then exposed overnight to X-ray film as outlined previously.

Polyacrylamide gel analysis also indicated that human IDP failed to sever the intradimer phosphodiester bond of a cyclobutane dimer present in an oligomer of 11 nucleotides. Oligonucleotides containing a modified cyclobutane dimer site were therefore established by photoligating two smaller oligonucleotides (Lewis and Hanawalt, 1982). This method of artificially generating this species was considered appropriate as it had been previously employed, along with SVP/CAP hydrolysis, to generate a trithymidylate marker of structure d-TpT<>dT from UV-irradiated poly(dA)·poly(dT) (Liuzzi and Paterson, in press). This marker had proved to be invaluable for the identification of modified cyclobutane dimers contained in excision fragments (this study) as well as for modified dimers produced in poly(dA)·poly(dT) by human IDP (Liuzzi *et al.*, manuscript in preparation). The structure generated by photoligation was, therefore, considered to be equivalent (or at least sufficiently analogous) to that produced upon severage of an intradimer phosphodiester bond by IDP.

The two oligonucleotides purchased for photoligation experiments, AGA-9 and AGA-8, together comprise the sequence of AGA-6 (i.e., if AGA-8 were to be ligated to the 3'-OH of AGA-9, AGA-6 would be generated). Prior to photoligation of these two species, a small amount of the oligonucleotide were each end-labelled with [^{32}P]. Since AGA-8 had been purchased in a 5'-phosphorylated form, this oligonucleotide first required dephosphorylation by calf alkaline phosphatase. Fifty pmol of AGA-8 was hydrolysed in a 20-µl reaction containing 50 mM Tris-HCl (pH 8.5), 0.1 mM EDTA, and 1 unit phosphatase for 1 hr at 37°C. The CAP was subsequently heat inactivated by incubating at 75°C for 10 min, and phenol/chloroform extracted to ensure the removal of all phosphatase activity. This 50 pmol of dephosphorylated AGA-8, as well as 50 pmol of AGA-9, were then each kinased in a 50-µl reaction mixture consisting of 10 mM Tris acetate (pH 7.0), 10 mM magnesium acetate, 50 mM potassium acetate, 2 µCi of [γ-^{32}P] ATP (1 mCi/ml; 3,000 Ci/mmol; Amersham), and 20 units of T4 polynucleotide kinase. Incubation was terminated after 1 hr at 37°C by the addition of EDTA (pH 8.0) to a final concentration of 50 mM. A small aliquot of one or both labelled oligonucleotide could then be added to a photoligation reaction, thus allowing easy assessment of the reaction outcome by HPLC or polyacrylamide gel electrophoresis. The retention time on the reverse-phase HPLC (Nova-Pak column), employing the gradient system which had been used for the separation of AGA-6 from its T^T-containing derivative, was established for both end-labelled species by collecting all 80 0.5-ml fractions and determining their radioactivity content by Cherenkov counting.

Efficient photoligation required the jutxapositioning of the 3'-thymidine of AGA-9 to the 5'-thymidine of AGA-8. This was achieved by first hybridizing these two oligonucleotides to their complementary oligomer, AGA-7. In initial experiments designed to test the efficiency of the photoligation reaction, 2 pmol of AGA-9, 2 pmol (~1 x 10^6 cpm) of [^{32}P] end-labelled AGA-8, and 2 pmol of AGA-7 were diluted in each of 6 1.5-ml polypropylene tubes to a final volume of 40 µl with ddH_2O, and supplemented with 10 µl of 0.5 M Na Phosphate and 50 µl of 0.1, 0.2, 0.5, 1, 2, or 4 M NaCl, to find the best annealing conditions. These 6 oligonucleotide solutions were heated to 65°C for 10 min, and subsequently allowed to cool to room temperature. The hybridization reaction was then left at 4°C overnight. In the morning, the hybridized oligonucleotides were irradiated as drops set atop a 10 x 10 cm piece of Parafilm 'M' Laboratory Film (American Can Company, Greenwich, CT) which was placed inside a 150-mm tissue culture dish. This dish was positioned on a bed of ice and, with an open lid, exposed to a fluence of 5.5 kJ/m^2 of 254-nm light. The resulting products of each reaction were analyzed by reverse-phase chromatography (utilizing the same column and gradient conditions used for establishing the retention times of [^{32}P] end-labelled AGA-8 and AGA•9). Eighty 0.5-ml fractions were collected and assessed for radioactive content. This analysis revealed a novel peak in addition to the [^{32}P] end-labelled AGA-8 and unincorporated [^{32}P] ATP present in the original reaction mixtures. It represented the highest percentage of total radioactivity in that reaction which contained a final NaCl concentration of 1 M. As the radioactivity in these samples had been determined by Cherenkov counting, this novel peak could be recovered and subjected to further examination to establish that it in fact represented the photoligation product.

To confirm that this novel peak contained photoligated AGA-9<>AGA-8, the material recovered from HPLC analysis of the 1 M NaCl hybridization/ photoligation reaction, was subjected to photochemical reversal. In order to perform this operation, the recovered sample was first taken to dryness in a rotoevaporator (to remove the acetonitrile) and resuspended in 1 ml of ddH_2O. The solution was then transferred to a 40-mm tissue culture dish, exposed to a total fluence of 5.5 kJ/m^2 of 254-nm light, and reinjected directly onto the HPLC. The regeneration of AGA-8 demonstrated that the novel peak had, in fact, contained photoligated oligomers. Furthermore, this material now served as an HPLC marker for the production of large quantities of this species.

As it had now been established that highest production of a photoligated species occurred when hybridization took place in 1 M NaCl, the above experiment was repeated using both end-labelled AGA-9 and AGA-8. Two pmol of [^{32}P] end-labelled AGA-9, 2 pmol of [^{32}P] end-labelled AGA-8, and 2 pmol of AGA-7 were hybridized in a 100-µl reaction mixture containing 50 mM Na Phosphate and 1 M NaCl. Subsequent hybridization and irradiation condition were identical to those outlined above. The irradiated material was analyzed by reverse-phase HPLC which, again, revealed a novel peak. Photoreversal of material contained in this novel peak, performed in a manner identical to that outlined above, regenerated both 5'-phosphorylated AGA-9 and AGA-8, establishing that it represented the photoligation product of these oligomers. In addition, this 5'-phosphorylated derivative of AGA-9<>AGA-8 provided a radioactively-labelled marker for subsequent kinasing of this photoligated species.

Having established (i) the NaCl concentration under which the most efficient photoligation occurred, (ii) a [^{32}P]-labelled marker for AGA-9<>AGA-8, and (iii) a radioactively-labelled marker for 5'p-AGA-9<>AGA-8, a large scale preparation of the photoligated oligonucleotides was prepared. Three hundred and sixty nmol of AGA-9, 90 nmol of AGA-8, 90 nmol of AGA-7, and 2 pmol of [^{32}P] end-labelled AGA-8 were diluted into a 20-ml volume containing 50 mM

Na Phosphate (pH 6.8) and 1 M NaCl in a 50-ml polypropylene tube. This reaction mixture was heated to 65°C for 10 min, allowed to cool to room temperature, and finally left at 4°C overnight. In the morning, the sample was transferred to a 150-mm tissue culture and irradiated, as indicated previously, with 5.5 kJ/m^2 of 254-nm light. The sample was reduced to ~0.5 ml in a rotoevaporator, and subsequently loaded onto the Nova-Pak reverse-phase column. The 0.5-ml fractions of effluent were collected into 80 1.5-ml polypropylene tubes. As the original hybridization reaction had been spiked with [^{32}P] AGA-8, the radioactive content of the samples was determined with Cherenkov counting to locate the AGA-9<>AGA-8-containing fractions. These were then pooled, taken to dryness in a rotoevaporator, and resuspended in 1 ml of ddH$_2$O. This sample was subsequently dialysed overnight against 2 *l* of ddH$_2$O, as outlined above, to reduce the concentration of triethylammonium acetate. Approximately 25 µg or 8.3 nmol of photoligated oligonucleotides were recovered.

The purified AGA-9<>AGA-8 required terminal phosphorylation prior to ligation to the pGEM vector sequence. This was accomplished in a manner analogous to 5'-phosphorylation of AGA-6. Two nmol of oligomer was kinased in a 1-ml reaction mixture consisting of 10 mM Tris acetate (pH 7.0), 10 mM magnesium acetate, 50 mM potassium acetate, 50 nmol unlabelled ATP, and 50 units of T4 polynucleotide kinase. The reaction mixture was incubated overnight at 37°C and was repeated 4 times in all. The following morning, one 1-ml reaction mixture, spiked with 1 x 10^4 cpm of [^{32}P]-AGA-9<>AGA-8, was injected onto the Nova-Pak reverse-phase HPLC column. Gradient conditions were identical to those used for isolating the photoligated molecules from their derivative oligonucleotides. As before, eighty 0.5-ml fractions were collected directly into 1.5-ml polypropylene tubes, and the radioactive content determined by Cherenkov counting. As a marker molecule was present, the fractions of phosphorylated material were retained and pooled from all 4 HPLC runs. These were subsequently taken to dryness in a rotoevaporator, resuspended in 1 ml of ddH$_2$O, and dialysed overnight against 2 *l* of ddH$_2$O. The final concentration of this modified dimer-containing 11-mer was 3 pmol/µl. Approximately 4 nmol in all were isolated.

C. **Ligation of Oligonucleotides to the pGEM Vector 2917-bp Fragment** The initial attempt to ligate AGA-4, 5'p-AGA-6, and AGA-7 to the 2917-bp pGEM-7Zf(-) fragment involved combining 8 pmol of each of these three oligonucleotides with 800 fmol of the 2917-bp pGEM fragment in a 1-ml reaction mixture containing 100 µl of 10 x ligase buffer (supplied with the T4 ligase and consisting of 0.5 M Tris-HCl (pH 7.5), 0.1 M $MgCl_2$, 0.1 M dithiothreitol, and 160 mM ATP) and 1 unit Weiss unit of T4 ligase (Promega). This mixture was incubated at 14°C overnight. As the presence of proteins interfered with agarose gel analysis, the following morning the 1-ml solution was phenol/chloroform-extracted and the DNA ethanol precipitated as outlined previously. The DNA pellet was collected by centrifugation and dried in a SpeedVac Concentrator. After resuspending in 20 µl of ddH_2O, the reaction products were assessed by minigel electrophoresis on a 0.8% agarose gel containing 0.5 µg/ml ethidium bromide, as described earlier. Because of the precautions taken, only generation of the desired substrate would result in a CCC molecule. However, analysis of the ligation reaction indicated an extremely low and insufficient yield of this CCC DNA. As a potential means of increasing this yield, the pGEM sequence and oligonucleotides were prehybridized prior to the addition of T4 ligase. Eight pmol of each AGA-4, 5'p-AGA-6, and AGA-7 were combined with 800 fmol of the 2917-bp pGEM fragment in a 1-ml reaction mixture containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 10 mM dithiothreitol. The mixture was then heated to 65°C in a circulating water bath for 10 min to denature all secondary structure. The waterbath was then adjusted to 14°C and the hybridizing mixture was allowed to cool gradually. The target temperature of 14°C was maintained for 1 hr before adding ATP to a final concentration of 16 mM, and 1 units of T4 ligase. Incubation was then continued overnight at 14°C. Analysis by agarose gel electrophoresis, following purification of the DNA by phenol/chloroform extraction and ethanol precipitation, confirmed that this procedure had greatly increased the production of CCC substrate DNA. This reaction was, therefore, scaled up to generate a useable amount of substrate DNA. For this purpose, 16 pmol of the 2917-bp pGEM-7Zf(-) vector sequence was mixed with 160 pmol of each AGA-4, AGA-7, and 5'-phosphorylated AGA-6 in a final volume of 20 ml containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 10 mM dithiothreitol. As done earlier, the mixture was heated to 65°C for 10 min before lowering the temperature of the waterbath to 14°C and allowing the hybridizing molecules to cool gradually. After maintaining 14°C for 1 hr, the reaction was supplemented with ATP to a final concentration of 16 mM, and 15 Weiss units of T4 ligase, and incubated overnight at 14°C. The following morning, the ligation mixture was phenol/chloroform-extracted, and the DNA ethanol precipitated. This ethanol precipitate was left at -20°C (at least overnight) until purification procedures could be initiated.

The above procedure was repeated twice more using instead of adding 5'p-AGA-6 to the hybridization mixture, either 160 pmol of 5'-phosphorylated T^T-containing AGA-6 or 160 pmol of 5'-phosphorylated AGA-9<>AGA-8.

D. Purification of Substrate Molecules by Low Melt Agarose Gel Electrophoresis and Electroelution A DNA pellet from one of the above ligation reactions was collected by centrifugation at 5000 rpm (Beckman AccuSpin FR benchtop centrifuge) for 10 min at 4°C. The ethanol was then decanted and the DNA was dried under vacuum in a SuperVac Concentrator. The dried pellet was resuspended in 100 µl of ddH_2O, supplemented with 50 µl an agarose gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol), and subsequently loaded (at 20 µl per lane) onto a 0.8% low-melt agarose (ultraPURE; BRL) gel containing 0.5 µg/ml ethidium bromide. A 1-µg sample of uncut pGEM vector was loaded in an outside lane and served as a control fro the position of CCC DNA. Electrophoresis was carried out, using TAE buffer in a Model H5 horizontal gel tank (BRL), at 20 V overnight at 4°C in the dark. In the morning, bands were visualized on a UV Transilluminator, and the CCC DNA band was cut from the gel with a scalpel. This gel slice was placed in a 2.5 cm-wide prepared dialysis bag (3 kDa MW cut-off) with approximately 500 µl of TAE, and the tubing ends were sealed with clips. The gel slice was gently pushed to one side of the tubing, which was then positioned in the center of a electrophoresis tank (at 4°C) with the gel slice closest to the negative electrode. A current of 150 V was then applied for 3 hr, after which time the polarity was then reversed for 1 min to free the electroeluted DNA from the wall of the dialysis membrane. The buffer surrounding the gel slice was transferred to a 1.5-ml polypropylene tube and subsequently extracted with phenol/chloroform to remove both the ethidium bromide and gel contaminants. The DNA was ethanol precipitated from the resulting aqueous phase after the addition of ammonium acetate to a final concentration of 1 M. The resulting suspension was left at -70°C for 1 hr before collection the DNA pellet by centrifugation. The pellet was then dried and resuspended in 500 µl ddH_2O.

All three DNA substrates were isolated in this manner. To further concentrate and purify this material, each DNA solution was passed through a Centricon-10 concentrator (Micon, Beverly, MA). Each sample was applied to the top reservoir of the device and spun at 4800*g* for 15 min in a fixed angle rotor (Sorvall SA-600 rotor; Sorvall RC-5C centrifuge). The sample was then washed twice. Each wash involved adding 500 µl of ddH_2O to the top reservoir and repeating the centrifugation. The unit was subsequently inverted and the cleaned concentrate was collected by spinning at 600*g* for 2 min. Purified substrates were stored at 4°C until further use. Approximately 1 ng of each substrate was isolated.

For simplicity, the three substrates will be referred to by code names: m-pGEM lacks a lesion; ID-pGEM refers to the substrate containing a cyclobutane dimer at the unique TT sequence within AGA-6; MD-pGEM contains the modified cyclobutane dimer.

II. Assessment of the Activity of DenV on Unaltered and Modified Cyclobutane Dimers

A. DenV Hydrolysis of UV-Irradiated CCC Plasmid DNA The DenV protein used in the experiments outlined below was isolated by John Chan in our laboratory using the procedure of Seawell *et al.* (1981). Prior to experimentation involving the pGEM/oligonucleotide constructs, the activity of various DenV preparations was first tested on UV-irradiated CCC DNA. For this purpose, 70 µg of unrestricted pGEM-7Zf(-) was diluted to 1 ml with ddH_2O and transferred to a 40-mm tissue culture dish. The solution was then exposed, with an open lid and with continuous mixing, to a total fluence of 2 kJ/m^2 of 254-nm light (lamp source as described previously). At an induction rate of 1.2×10^{-5} cyclobutane dimers/nt/J/m^2 (this study), this would generate, on average, 72 cyclobutane dimers per plasmid molecule. The activity of each DenV preparation was then tested on this lesion-containing substrate and compared to the activity on non-irradiated plasmid.

Three hundred and fifty ng (~180 fmol) of irradiated or untreated plasmid DNA was incubated in several 20-µl reaction mixtures containing 32 mM Tris-HCl (pH 7.5), 9.6 mM EDTA, and varying amounts (0.1, 0.5, 1.0, 2.5, or 5 µl of three different supplied preparations) of DenV, for 15 min at 37°C. NaOH was subsequently added to a final concentration of 0.2 N and the mixture held for a further 30 min at 37°C. [Whereas the glycosylase activity of DenV is very active under the initial reaction conditions, the AP endonuclease activity is less so. Although the addition of NaOH inhibits any further DenV hydrolysis, these alkali conditions stimulate the generation of single-strand breaks at existing AP sites by β-elimination (Nakabeppu and Sekiguchi, 1981).] The reaction was supplemented with SDS to a final concentration of 0.01%. Prior to analysis by agarose gel electrophoresis, the mixture was supplemented with 2.5 µl of agarose gel loading dye. Electrophoresis was carried out using a 0.8% agarose minigel which contained 0.5 µg/ml ethidium bromide and was submersed in TAE buffer, at 50 V for 2 hr at room temperature. Specific incision by DenV could be detected as a conversion from CCC to open-circle DNA in the case of the UV-irradiated substrate, without effect on the untreated plasmid. One of the three preparations was active on UV-irradiated DNA while causing no alteration of unirradiated DNA.

One unit of DenV is defined as the amount of enzyme required to nick 1 fmol of 5.5×10^6 Da of CCC DNA (which contains an average of 2 cyclobutane dimers/molecule) in 15 min at 37°C (Seawell *et al.*, 1981). Therefore, the amount of DenV required to nick 20% of the CCC DNA in this reaction is approximately 36 units. As it required 0.5 µl of the active DenV preparation to nick 20% of UV-irradiated DNA, this DenV preparation has a concentration of ~70 units/µl.

B. DenV Hydrolysis of pGEM-Derived Constructs Containing at a Defined Site (i) No Lesion, (ii) an Intact Cyclobutane Dimer, and (iii) a Cyclobutane Dimer with a Severed Intradimer Phosphodiester Backbone Once an active preparation of DenV had been established, the ability of DenV to nick the three prepared substrates was ascertained. Because of the limited quantities of these substrates, they were cut with a restriction enzyme and end-labelled.

Five hundred fmol of each substrate was restricted in a 50-µl reaction containing 10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate, 50 mM NaCl, and 5 units of *Pvu*II (Pharmacia). The reaction mixtures were incubated at 37°C for 1 hr and subsequently phenol/chloroform-extracted and ethanol precipitated. After 30 min at -70°C, the DNA pellets were collected by centrifugation (Eppendorf model 5415 microfuge, Brinkmann) at 4°C for 10 min. After drying under vacuum, each DNA sample was resuspended in 20 µl of ddH_2O. *Pvu*II digestion generates two fragments, of 373- and 2567-bp. Prior to end-labelling of these fragments, each sample was first dephosphorylated in a 100-µl reaction containing the 2 pmol of restricted DNA, 50 mM Tris-HCl (pH 8.5), 0.1 mM EDTA, and 2 units of CAP for 1 hr at 37°C. These protein-containing reactions were again phenol/chloroform-extracted, and the DNA was precipitated with ethanol. The DNA pellets were collected by centrifugation, dried under vacuum, and each resuspended in 43 µl of ddH_2O. Each sample of dephosphorylated *Pvu*II cut DNA was then supplemented with 5 µl of 10 x kinase buffer (consisting of 100 mM Tris-acetate, 100 mM magnesium acetate, and 500 mM potassium acetate), 1 µl of [γ-^{32}P] ATP (10 mCi/ml; 3000 Ci/mmol; NEN), and 1 µl T4 polynucleotide kinase (10,000 units/ml; Pharmacia). These reaction mixtures were incubated overnight at 37°C. The following morning, each sample was again phenol/chloroform extracted and subsequently ethanol precipitated, dried, and resuspended in 10 µl of ddH_2O. As unincorporated nucleotides stay in solution during ethanol precipitation, this procedure reduced the radioactivity of each sample approximately 10-fold. Each 500 fmol of restricted end-labelled substrate had a radioactive content of ~2.5 x 10^5 cpm.

Approximately 15,000 cpm of each restricted end-labelled DNA substrate was employed in each DenV experiment. This corresponds to ~30 fmol of 373-bp fragment (which, when derived from ID-pGEM or MD-pGEM, contains the cyclobutane dimer). It would, therefore, require 30 units, or < 0.5 µl, of the DenV preparation described above to nick all of the cyclobutane dimers contained in a 15,000 cpm sample of these end-labelled restricted ID-pGEM or MD-pGEM preparations in 15 min at 37°C.

In a similar manner as for UV-irradiated plasmid, ~30 fmol of *Pvu*II restricted, [^{32}P] end-labelled m-pGEM, ID-pGEM, or MD-pGEM was incubated in a 5-µl reaction containing 32 mM Tris-HCl (pH 7.5), 9.6 mM EDTA, 35 units of DenV. After 30 min at 37°C, 1.3 µl of 1 N NaOH was added and incubation was the continued for an additional 30 min at 37°C. The reactions were supplemented with 0.7 µl of 0.1% SDS and 2 µl of Stop Buffer (consisting of 0.2% bromophenol blue, 0.2% xylene cyanol, and 90% formamide; Pharmacia), and set aside for analysis by polyacrylamide gel electrophoresis.

In a second series of DenV experiments, instead of assessing the activity of this enzyme on the artificially constructed modified cyclobutane dimer-containing construct, MD-pGEM, ID-pGEM was pretreated with human IDP to generate an authentic modified site. Thirty fmol (1.5 x 10^4 cpm) of *Pvu*II-cut end-labelled ID-pGEM was incubated in a 50-μl reaction mix containing 25 mM NaAc (pH 5.5), 2.5 mM $MgCl_2$, 1 mM β-mercaptoethanol, and 5 ng of human IDP at 37°C for 1 hr. After heat denaturation of the IDP protein by heating to 100°C, the substrate was purified by phenol/chloroform extraction and ethanol precipitation. The DNA was resuspended in 3.5 μl of ddH_2O, and supplemented with 1 μl of 5 x DenV Buffer [consisting of 160 mM Tris-HCl (pH 7.5) and 48 mM EDTA] and 0.5 μl of DenV (70 units/μl). Following incubation at 37°C for 30 min, the reaction was supplemented with NaOH, incubated, and stopped with SDS and Stop Buffer exactly as outlined above. In parallel with this experiment, DenV hydrolysis of *Pvu*II-cut [^{32}P]-labelled ID-pGEM and m-pGEM, neither of which had been pretreated with DenV, was repeated as described previously.

As earlier results from our laboratory have demonstrated that although photolyase can recognize a modified cyclobutane dimer site, it does so with greatly reduced efficiency (Liuzzi and Paterson, in press). Approximately a 10-fold higher concentration of enzyme is required to photoenzymatically reverse these lesions as compared to intact cyclobutane dimers. Therefore, as the earlier DenV experiments employed only a two-fold excess of that enzyme required to incise all of the cyclobutane dimers present under the reaction conditions used, it was deemed necessary to repeat these reactions with a much higher concentration of DenV enzyme. Therefore, 30 fmol of each *Pvu*II-cut end-labelled substrate was incubated in a 20-μl reaction mixture consisting of 4 μl of 5 x DenV buffer, 2 μl of the substrate molecule, 4 μl of ddH_2O, and 10 μl of DenV (700 units; final concentration of 35 units/μl) for 2 hr at 37°C. The combination of increased incubation time and augmented enzyme provide an 80-fold excess of DenV over that required to incise every cyclobutane dimer present in the ID-pGEM preparation. After the incubation period was complete, these reaction mixtures were supplemented with NaOH, incubated, and stopped with SDS precisely as outlined above. Because of the larger reaction volume, these samples were reduced to ~5 μl in a SpeedVac Concentrator prior to the addition of 2 μl of Stop Buffer.

C. Polyacrylamide Sequencing Gel Analysis of DenV Cleavage Products A 7%

polyacrylamide sequencing gel was prepared by dissolving 57 g of urea, in a 200-ml Erlenmeyer flask, in 21 ml of a 40% acrylamide solution [containing 38 g acrylamide (ultrapure quality; International Biotechnologies, Inc.) and 2 g *N,N'*-methylene bisacrylamide (ultraPURE; BRL) made up to 100 ml with ddH_2O], 12 ml of 10 x TBE buffer (consisting of 0.89 M Tris base, 0.89 M boric acid, and 20 mM EDTA), and 20 ml of ddH_2O by placement in a 37°C waterbath. The volume of the solution was then adjusted to 120 ml and subsequently filtered through Whatman No. 1 paper into a 250 ml side-arm flask. The flask was corked and then attached to a vacuum to degas the acrylamide solution. After degassing for 10 min, 120 µl of a fresh 10% ammonium persulfate solution (made with ddH_20) and 12 µl of TEMED were added with gentle mixing. The resulting mixture was poured, using a syringe, between two clean siliconized vertical glass plates (35 cm x 40 cm and 20 x 38 cm) which were separated by 1 mm spacers on three sides. A shark's tooth comb (1 mm thick) was inserted into the top of the glass plates to produce wells for loading samples. After the gel had polymerized, the bottom spacer was removed from the glass plates, and the gel was placed into the sequencing electrophoresis apparatus (Model STS45, International Biotechnologies, Inc.). Both the top and bottom tanks of this apparatus were then filled with TBE. After removing all air between the gel and the buffer contained in the apparatus, the gel was prerun for 15 min at 70 W (~2000 V; Model 3000/300 power supply; BioRad) with 5 µl of Stop Buffer (described above) in 2 of the 40 lanes. The nine samples described above, as well as 15,000 cpm of each untreated *Pvu*II-restricted end-labelled substrate in a 5-µl volume containing 50% Stop Buffer, were heated to 80°C for 2 min (Haake L water bath; Berlin, Germany), and 5 µl of each subsequently loaded onto the gel, separated by at least one lane. In addition, three 2-µl samples of [^{32}P] end-labelled marker DNA (described below) were loaded onto the two outside lanes and the center lane. Separation of the various length molecules was achieved by applying 70 W for ~1 hr (until the first dye front had migrated to within 2 cm of the bottom of the gel).

A marker DNA ladder was purchased from Sigma as a HaeIII digest of pBR322 DNA. It consists of 22 fragments ranging from 8 to 587 bp (8 / 11 / 18 / 21 / 51 / 57 / 64 / 80 / 89 / 104 / 123 / 124 / 184 / 192 / 213 / 234 / 267 / 434 / 458 / 504 / 540 / 587 bp). Thirty-five pmol of this DNA was first dephosphorylated in a 50-µl reaction containing 50 mM Tris-HCl (pH 8.5), 0.1 mM EDTA, and 1 unit of CAP. This mixture was incubated for 1 hr at 37°C, and subsequently phenol/chloroform-extracted and ethanol precipitated as described previously. After recovery of the DNA pellet by centrifugation, it was dried under vacuum and resuspended in 38 µl of ddH_2O. This DNA solution was then supplemented with 5 µl of 10 x One-Phor-All Buffer Plus (100 mM Tris-acetate, 100 mM magnesium acetate, and 500 mM potassium acetate; Pharmacia), 5 µl [γ-^{32}P]ATP (10 mCi/ml; 3000 mCi/mmol; NEN), and 1 µl of T4 polynucleotide kinase (10 units/µl). After incubating overnight at 37°C, 50 µl of Stop buffer and 200 µl of ddH_2O were added. The unused portion of these markers were stored at -70°C.

After electrophoresis of the polyacrylamide gel was complete, it was transferred to Whatman filter paper, wrapped with plastic film, and exposed overnight on diagnostic X-ray film (35 x 43 cm; Kodak Canada Inc.). Following film development, the relative amount of DNA representing each individual band was assessed using an LKB UL Ultrascan XL Laser Densitometer (LKB-Produkter, Bromma, Sweden).

III. Assessment of the *In Vitro* Activity of *E. coli* DNA Polymerase I on Unaltered and Modified Cyclobutane Dimers

A. Substrate Preparation The pGEM constructs were assembled in an orientation such that if replication begins from the reverse sequencing primer, the polymerase would encounter the cyclobutane dimer inserted into the multiple cloning site. To make use of this system, the substrate DNA was first restricted site such that *in vitro* replication would produce a run-off product of a discrete and convenient size. Substrate cut with three different restriction enzymes was used in separate experiments, *Apa*I (Promega), *Acy*I (Promega), and *Pvu*II (Pharmacia). Approximately 1.2 pmol of m-pGEM, ID-pGEM, and MD-pGEM were each restricted in a 50-µl reaction containing 5 µl of the supplied 10 x reaction buffer, 0.1 mg/ml BSA, and 20 units of *Apa*I, *Acy*I, or *Pvu*II. After incubation at 37°C for 1 hr, the nine digestion reactions were phenol/chloroform extracted and ethanol precipitated. The DNA pellets were recovered by centrifugation, dried, and each resuspended in 50 µl of ddH_2O.

B. Annealing of the Reverse Sequencing Primer to the Substrate Fifty fmol of restricted m-pGEM, ID-pGEM, and MD-pGEM were each incubated in 0.4 M NaOH, in a total volume of 10 µl, for 10 min. These denaturation reactions were then supplemented with 3 µl of 3 M Na acetate (pH 4.8) and the volumes increased to 20 µl with ddH_2O. After the addition of 60 µl of ice-cold 100% ethanol, the mixtures were incubated on dry ice for 30 min. The DNA was then collected by centrifugation, washed once with 70% ethanol, and taken to dryness under vacuum. The pellets were redissolved in 14 µl of annealing buffer [containing 0.1 M NaCl, 10 mM Tris-HCl (pH 7.8), and 1 mM EDTA] containing 2 ng of the 17-mer reverse sequencing primer (Promega). (The reverse sequencing primer is an oligomer which anneals at a specific location to all M13-derived plasmids. Replication can then be primed from the free 3-OH group.) After incubation at 37°C for 20 min, these hybridization mixtures were left at room temperature for 1 hr.

C. *In Vitro* Replication Each 14-µl hybridization mixture was supplemented with 2.5 µl of 10 x polymerase buffer [containing 0.6 M Tris-HCl (pH 7.5), 0.1 M $MgCl_2$, and 0.1 M β-mercaptoethanol; Promega], 2.5 µl deoxynucleotide mixture (2 mM dATP, 2 mM dGTP, 2 mM dCTP, and 100 µM dTTP; all purchased from Promega), 5 µl [α-^{32}P]TTP (10 mCi/ml; 3000 Ci/mmol; Amersham), and 10 units of *E. coli* DNA polymerase I (Promega), and subsequently incubated at 12°C for 2 hr in a cooling circulating water bath. The reactions were terminated by the addition of 5 µl of Stop buffer (described above), and heated for 2 min at 80°C. The radioactive replication products were analyzed by loading 2 µl of each reaction on a 7% denaturing sequencing polyacrylamide gel, as indicated above.

IV. Assessment of the *In Vitro* Activity of SP6 RNA Polymerase on Unaltered and Modified Cyclobutane Dimers

A. Substrate Preparation Initiation of transcription from the SP6 promoter places the cyclobutane dimer, which occurs in ID-pGEM and MD-pGEM, on the transcribed strand. For ease of analysis of the products of *in vitro* transcription, all three substrates were first cut with a restriction enzyme such that a reasonable length run-off transcript would be produced (in the absence of a premature stop). Substrates were cut with one of *Apa*I, *Acy*I, or *Pvu*II in precisely the same manner as described above for substrate preparation for *in vitro* replication, with one important exception. Since *Apa*I leaves 3'-

protruding single-stranded ends, these needed to be blunt-ended before attempting *in vitro* transcription as their presence often generates aberrant transcripts. Prior to phenol/chloroform extraction of the *Apa*I restriction digests, they were supplemented with 1 µl of a deoxynucleotide solution (containing 2 mM dATP, 2 mM dGTP, 2 mM dCTP, and 2 mM dTTP) and 10 units of T4 DNA polymerase (Promega), and the combined mixture was subsequently incubated at 37°C for 10 min. These, along with the *Acy*I and *Pvu*II digests, were then phenol/chloroform extracted and ethanol precipitated. The DNA pellets were recovered by centrifugation, dried, and each resuspended in 50 µl of ddH_2O.

B. *In Vitro* Transcription using SP6 RNA Polymerase Fifty fmol of restricted m-pGEM, ID-pGEM, and MD-pGEM were each incubated in a 20-µl reaction mixture containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 40 units of RNasin (Promega), 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 12 µM UTP, 3 µl [α-^{32}P] UTP (10 mCi/ml; 3000 Ci/mmol; Amersham), and 15 units SP6 RNA polymerase (Promega) for 1 hr at 37°C. The reaction was terminated with 5 µl of stop buffer and heated for 2 min at 80°C. The radioactive transcription products were analyzed by loading 2 µl of each reaction on a 7% denaturing sequencing polyacrylamide gel, as outlined previously.